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Studies on Growth Factors for *Sporotrichum Schenckii*

By

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(Received Nov. 8, 1951)

Sporotrichum Schenckii is an imperfect fungus causing disease in man and animals. The organism is dimorphic, having a yeast phase in the tissues of man and animals and a mycelial phase on artificial media as well as on plant material in nature. As yet the yeast phase has not been grown on chemically defined media (7). (In a medium containing casein hydrolyzate, however, yeast phase has recently been obtained (4).) As chemically defined media are desired for the study of growth factor requirements the present work was devoted to the mycelial phase, grown on a simple basic medium. — The morphology and behaviour of *S. Schenckii* were recently described in detail in a monograph by Nordén (7).

Material and Methods

S. Schenckii, strain 2079, from the collection of Dr. N. F. Conant, Duke Univ., Durham, N. Carolina, U.S.A., was used throughout this investigation. This strain had originally been isolated from a human case of sporotrichosis.

The following glucose-asparagine-medium was used as the *basic medium*:

Glucose	50 g.
Asparagine	2
KH ₂ PO ₄	1.5
MgSO ₄ · 7H ₂ O	0.5

Trace elements in p.p.m.: 0.01 B, 0.01 Mn, 0.07 Zn, 0.01 Cu, 0.05 Fe, 0.01 Mo

Distilled water to make 1000 ml.

The water used was treated with a cation exchanger (IR-100) in order to remove copper. The pH of the medium was adjusted to 5.6—5.7 by adding twenty drops of 2.5 N NaOH per litre. (All pH determinations were made by Hellige pH-meter No. 7040 fitted with a glass electrode).

The sugar, asparagine, salts, and growth substances were standard preparations delivered by the following companies: glucose, chemically pure, by R. Grave AB, Stockholm, asparagine, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, H_3BO_3 , $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, ammonium molybdate and inositol by Kebo AB, Stockholm, KH_2PO_4 and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ by Coleman & Bell Co., Norwood, O., U.S.A., $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ by J. D. Riedel-E. de Haën, A.-G., Berlin, thiamin, pyridoxine, riboflavin, indole acetic acid, and 2-methyl-1,4-naphthoquinone by F. Hoffman-La Roche & Co., Basle, pantothenic acid by Bios Laboratories, N.Y., biotin by Merck & Co., Rahway, N.J., and p-amino-benzoic acid, for chemical purposes, by Eastman Kodak Co., Rochester, N.Y.

Glassware (except pipettes) was cleaned by brushing with dish-washing powder. After rinsing with tap water, powder residues were removed with hydrochloric acid (about 5 N). The glassware was then again thoroughly rinsed with tap water, distilled water and finally distilled copper-free water. — Pipettes were treated with dichromate cleaning solution, then kept for several hours in tap water, rinsed in distilled water and finally distilled copper-free water.

The experiments were made in 200 ml. Erlenmeyer flasks of Pyrex glass. As recommended by Foster (5, p. 61) 40 ml. of the culture medium were added to each flask. The flasks were then twice sterilized by steaming, 25 minutes each time with an interval of 22—24 hours. — Pipettes were sterilized in air of 150—160° C. for two hours.

A conidia suspension was used as the inoculum. This suspension was obtained by growing the organism in a liquid medium containing 1 per cent peptone and 4 per cent glucose. After 5—12 days the culture was filtered through four layers of gauze, thereby almost completely eliminating the mycelium from the conidia and giving the desired suspension of conidia.

The conidia were freed from the peptone-glucose medium by centrifugation and washing. 1.5—3.0 ml. of the suspension — the exact amount depending upon the age of the culture — was aseptically pipetted into Klett-Summerson standard colorimeter test tubes, which had been cut off at the 10 ml. mark in order to fit the cups of a Corda centrifuge in which they were mounted with the aid of cotton wool. Centrifugation was carried out for three minutes at 2500 r.p.m. at room temperature. The supernatant was decanted off, and the basic medium added to the 5 ml. mark. After resuspending the conidia were again centrifuged, and the process of decanting and resuspending repeated twice.

The suspension of conidia in basic medium thus obtained was used as the inoculum. The conidia density was determined by the turbidity as measured in a Klett-Summerson photoelectric colorimeter using a blue filter No. 42, 400—465 m μ . In the first experiment ten drops of suspension, giving a colorimeter reading of 180, were used as the inoculum for each flask. In all later experiments, the number of drops needed to give about the same inoculum per flask as in the first case, was calculated from the colorimeter reading. This turbidity reading had been shown to be a linear function of the conidia density.

The cultures were left for 10 or 20 days, except when growth was determined as a function of time. In order to have the cultures well aerated and avoid concentration gradients in the medium, the flasks were placed on a shaking table of the reciprocal type (1), set at 100—120 2-cm. movements per minute. The cultures were kept in a dark room at 22—23° C.

At the end of the experiment the dried mycelium was weighed. Since the fungus is pathogenic, it was killed by autoclaving at 120° C. for 20 minutes. It was freed from the culture medium by filtering through a Pyrex 172124 glass filter crucible of 36 mm. diameter, and washing, first with distilled water and then with a small amount of 95 per cent alcohol. The crucibles were dried at 100—105° C. for 90 minutes and weighed after cooling in a dessiccator. — Before filtering off the fungus, the crucibles were treated in the same manner as above and their weight determined. — All experiments were made in duplicate.

Experiments

A. *Experiments with thiamin, pyridoxine, pantothenic acid, inositol, and biotin. Influence of pH*

The following concentrations of growth factors were used in these introductory experiments:

Thiamin	1 μ g. per 40 ml. flask
Pyridoxine	— 1
Pantothenic acid	1
Inositol	100
Biotin	0.01

Experiments were made with each one of these factors singly, with all except thiamin together, and with a complete mixture of them. The growth was determined after 10 and 20 days. In this connection, inoculum from the same peptone-glucose culture was used for two series with different initial

Table 1. *Effect of separate additions of thiamin, pyridoxine, pantothenic acid, inositol, and biotin (I—V) on growth of S. Schenckii.* Age of inoculum: 10 days. Initial pH: 4.4. Time of incubation: 10 days.

Medium	Additions per flask of						
	—	I 1 µg.	II 1 µg.	III 1 µg.	IV 100 µg.	V 0.01 µg.	I + II + III + + IV + V
Dry weights	1.8	154.9	7.8	0.5	9.2	3.1	151.1
in mg.	1.8	132.6	5.8	3.4	9.7	2.9	—

pH. The values of table 1 were obtained at an initial pH of 4.4, whereas in the experiment accounted for in table 2 (as well as in all later experiments) the cultures were started at a pH of 5.7.

An examination of tables 1 and 2 shows that thiamin gives a pronounced increase in growth. None of the other factors gives values differing greatly from the controls, neither does a mixture of them produce increased growth. According to table 1 a slight effect could possibly be ascribed to inositol but this could not be confirmed in later experiments (cf. table 3). — The higher pH value seems to improve growth.

The appearance of the cultures varied during the experimental period in the following manner. When *S. Schenckii* was cultured on the basic medium (or the basic medium + the growth factors investigated, except thiamin) no or almost no growth was observed over a period of about 30 days. When an optimal amount of thiamin was added, the medium became white and turbid after two days. Microscopic examination revealed the turbidity to be caused by fungus mycelium which appeared submerged in the medium, but not on the surface. After 6—8 days dark brown material was found in the medium and gathered especially along the periphery of the surface, close to the glass

Table 2. *Effect of additions of a) thiamin, b) pyridoxine, pantothenic acid, inositol, and biotin and c) = a) + b).* Concentrations as in table 1. Age of inoculum: 10 days. Initial pH: 5.7. Time of incubation: 10 and 20 days respectively.

Medium	Additions per flask of			
	—	a	b	c
Dry weights	2.0	185.3	2.5	179.6
in mg	—	188.1	3.3	179.0
10 days	—	180.9	—	—
Dry weights	—	283.4	1.5	—
in mg.	—	290.3	1.6	—
20 days	—	—	1.4	—

wall. This material consisted almost entirely of conidia. Up to this time the colonies had had a light colour, but from then on they became increasingly brown-black due to the formation of conidia throughout the medium.

B. Influence of thiamin and inositol in combination

The following experiment was made to elucidate further whether inositol alone or in combination with thiamin has any stimulating effect on the growth of *S. Schenckii*. Thiamin was given in the concentrations 0, 0.1 and 1 $\mu\text{g.}$ per flask; to each of these concentrations inositol was added in amounts of 0, 100, 500, and 1000 $\mu\text{g.}$ per flask. The results are shown in table 3. The growth increase seems to be due solely to the thiamin.

Table 3. *Influence on growth of thiamin and inositol in different combinations.* Age of inoculum: 9 days. Initial pH 5.7. Time of incubation: 10 days.

Additions per flask of		Dry weights in mg.	
thiamin $\mu\text{g.}$	inositol $\mu\text{g.}$		
—	—	2.8	0.5
—	100	3.6	3.6
—	500	3.7	1.8
—	1000	2.7	4.4
0.1	—	54.9	56.6
0.1	100	57.5	60.0
0.1	500	56.6	56.6
0.1	1000	57.8	41.6
1	—	284.3	—
1	100	192.1	187.8
1	500	199.7	203.6
1	1000	191.9	206.9

C. Significance of different thiamin concentrations

In order to elucidate in which concentration thiamin is required for optimal growth, experiments were made with the following concentrations: 0.01, 0.05, 0.1, 0.5, 1, 2, 5, and 20 $\mu\text{g.}$ per flask. The results of two different series are given in table 4. The age of the inoculum was 7 (a) and 8 (b) days respectively and the cultures were harvested after 10 days.

The dry weights obtained after 20 days exceeded those after 10 days with 25—50 per cent. The growth curve showed the same general features in both cases, however, with very slight growth at low concentrations of thiamin, then a rapid increase up to a concentration of about 0.5 $\mu\text{g.}$ per flask, where an optimum is reached and any further addition of thiamin has no effect on the growth.

Table 4. *Growth as related to different concentrations of thiamin. Age of inoculum: a: 7 days, b: 8 days. Initial pH: 5.7. Time of incubation: 10 days.*

Medium	Additions of thiamin per flask								
	—	0.01 μ g.	0.05 μ g.	0.1 μ g.	0.5 μ g.	1 μ g.	2 μ g.	5 μ g.	20 μ g.
a. Dry weights in mg.	3.9	—	—	135.3	172.0	188.5	—	143.6	—
	—	—	—	123.6	183.2	198.1	—	136.8	—
b. Dry weights in mg.	3.3	2.8	8.6	12.2	187.2	162.9	172.0	152.8	182.6
	2.1	2.8	8.1	12.2	188.5	159.0	204.9	219.2	178.6

D. *Growth in relation to time at different thiamin concentrations*

At suboptimal concentrations of thiamin growth went on for 5—6 days and then stopped, since the thiamin became the limiting factor for the mycelium production. The following experiments were made to elucidate further the time-growth relations at different thiamin levels. The thiamin amounted to 0.05, 0.1 and 1 μ g. per flask, and growth was determined in duplicate tests after 3(4), 6, 9, 12, and 15 days respectively. The results are shown in tables 5 and 6.

Table 5. *Growth in relation to time with additions of thiamin of 0.1 μ g. and 1 μ g. per flask. Age of inoculum: 8 days. Initial pH: 5.7. Control (15 days): 1.9 mg.*

Thiamin added per flask	Dry weights in mg. after (days)				
	4	6	9	12	15
0.1 μ g.	20.0	41.5	47.7	46.0	41.5
	14.9	44.6	48.7	46.2	47.7
1 μ g.	11.0	127.2	170.5	202.6	230.9
	20.8	131.2	166.8	206.6	222.9

Table 6. *Growth in relation to time with additions of thiamin of 0.05 μ g. and 0.1 μ g. per flask. Age of inoculum: 6 days. Initial pH: 5.7. Control (12 days): 3.7 mg.*

Thiamin added per flask	Dry weights in mg. after (days)				
	3	6	9	12	15
0.05 μ g.	4.8	4.9	10.6	9.9	—
	10.7	11.4	10.7	10.7	—
0.1 μ g.	11.7	17.5	18.2	19.3	17.1
	10.9	16.8	20.0	20.0	17.0

Only at the 1 μ g. per flask level was there enough thiamin to allow an increase in dry weight for the whole experimental time. At the lower levels growth stopped after 5—6 days, and the dry weight was maintained at an almost constant value for the rest of the incubation time. — As in previous experiments consistent results have been obtained only within series inoculated from the same stock suspension.

E. Influence of other substances

Experiments were made with additions of riboflavin amounting to 1, 5, 20, 50, and 100 μ g. per flask respectively. None of these concentrations showed any effect. Also p-amino-benzoic acid (2 μ g. per flask) and indole acetic acid (500 and 1000 μ g. per flask) seemed to be without effects on growth.

Additions of 2-methyl-1,4-naphthoquinone, which has been shown to possess great vitamin K properties (12), did not show any pronounced effect in the concentration range of 10—200 μ g. per flask. Also when thiamin (1 μ g. per flask) was combined with varying amounts of this vitamin K preparation (50, 100 and 200 μ g. per flask) no influence on growth was shown. A peculiar effect was obtained, however, since the pigmentation of the fungus spores as well as of the medium decreased with an increase in vitamin K.

Discussion

Since Schopfer 1934 (11) found thiamin to be essential for *Phycomyces Blakesleeanus*, an increasing number of fungi have been found to require this vitamin. (12, page 107). Robbins and Kavanagh (10) have given a review of vitamin deficiencies of filamentous fungi in which the growth of some species of the genus *Sporotrichum*, according to Oyama (9), is said to be stimulated by synthetic thiamin as well as by a concentrate of rice polishings when added to a basic medium of sodium chloride, ammonium lactate, disodium phosphate, asparagine and sugar. Other species of *Sporotrichum* were only stimulated by the rice polishings. As to the growth of *S. Schenckii* it was enhanced in both cases, although the rice polishings were more effective. This result seems to indicate some more essential growth factor in the rice polishings. As far as the author knows no other growth factors have as yet been shown to be essential for *S. Schenckii*, and of the growth factors used in this investigation only thiamin has given a clear-cut increase of growth. (Penicillin, although not a growth factor in the common sense, was

recently shown to stimulate *S. Schenckii* (8).) — In 1943 Burkholder and Moyer (2) confirmed the essentiality of thiamin for the growth of *S. Schenckii*. The stimulating effect on this fungus was recently assumed to be due to the pyrimidine nucleus (3).

The course of the growth curve as related to the thiamin concentration (exp. C) showed the following characteristics. Growth was very slightly affected by as small amounts of thiamin as $0.05\ \mu\text{g}$ per flask. With rising concentration a rapid increase in dry weights was noticed, growth being most vigorously stimulated in the concentration range of $0.1\text{--}0.5\ \mu\text{g}$. per flask. At a concentration of $1\ \mu\text{g}$. per flask growth had become optimal and a further increase in thiamin did not cause any significant variation of growth in either direction.

The most varying results were obtained with a thiamin concentration of $0.1\ \mu\text{g}$. per flask. The following dry weights in mg. have been found after ten days in different experiments: 135.3, 123.6 (age of inoculum 7 days), 12.2, 12.2 (8 days), 54.9, 56.6 (9 days), 15.5, 15.5, 14.1, 17.1, 14.4 (5 days). The cause of the variability may be partly attributed to the fact that $0.1\ \mu\text{g}$. per flask lies within the most rapidly sloping portion of the growth curve. Another explanation is the inequality of inoculum. Such an inequality might be due to variations in the age of the glucose-peptone cultures, which might give conidia in different states of development and varying amounts of accessory substances, including thiamin. The good agreement between cultures within the same series and the less satisfactory agreement between results from different series, which occur also at other concentration levels than $0.1\ \mu\text{g}$. per flask, seem to speak in favour of the latter explanation, while at suboptimal additions of thiamin the slope of the curve undoubtedly plays a definite role.

When growth was determined as a function of time (exp. D), at optimal amounts of thiamin the dry weights showed an ever increasing value during the entire experimental period. At suboptimal concentrations, however, growth stopped after 5—6 days, because the thiamin concentration had a limiting effect, and the growth-time curve showed a flat portion.

Fries (6) showed the quotient $\frac{\text{mycelium produced}}{\text{thiamin added}}$ to be a measure of the effectivity of thiamin, providing this substance is the only substance limiting growth. The quotient thus obtained varied with the experimental conditions and with the species investigated but lay between the border values of $0.5 \cdot 10^6$ and $2.5 \cdot 10^6$ and within the same species it was usually constant. — If this economic coefficient is computed from the results given in table 6, the $0.05\ \mu\text{g}$. per flask level gives a value of $0.14 \cdot 10^6$ and the $0.1\ \mu\text{g}$. level a value of $0.16 \cdot 10^6$. The corresponding computation on the $0.1\ \mu\text{g}$. level in

table 5 gives $0.46 \cdot 10^6$. The agreement between the quotients derived from table 6 supports the theory, that thiamin is the sole limiting factor in these experiments. The variation of the quotients for the 0.1 μ g. levels in tables 5 and 6 seems to be due to the variability mentioned above between the results of different series.

Summary

Growth factors for *Sporotrichum Schenckii* have been studied. Thiamin had a pronounced influence on growth as reflected in the dry weights of the cultures. Growth was studied in its relation to time and to thiamin concentration. A concentration of 1 μ g. per 40 ml. flask was shown to be sufficient for continuous growth during the entire experimental period. The economic coefficient $\left(\frac{\text{dry weight produced}}{\text{thiamin added}} \right)$ is computed for suboptimal additions of thiamin, where growth stops after 5—6 days.

No influence on growth was observed from pyridoxine, pantothenic acid, inositol, biotin, riboflavin, p-amino-benzoic acid, indole acetic acid, and 2-methyl-1,4-naphthoquinone (vitamin K).

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References

1. Algéus, S.: Studies on the cultivation of algae in artificial light. — *Physiol. Plant.* 4: 742. 1951.
2. Burkholder, P. R. & Moyer, D.: Vitamin deficiencies of fifty yeasts and molds. — *Bull. Torrey Bot. Club* 70: 372. 1943.
3. Drouhet, E. & Mariat, F.: La pyrimidine, facteur de croissance pour les *Sporotrichum*. — *Ann. l'Institut Pasteur* 79. 1950.
4. — — Sur le déterminisme du développement in vitro de *Sporotrichum schenckii* sous la forme levure. — *Compt. rend. (Paris)*, 233: 433. 1951.
5. Foster, J. W.: *Chemical activities of Fungi*. — New York. 1949.
6. Fries, N.: Über die Bedeutung von Wuchsstoffen für das Wachstum verschiedener Pilze. — *Symb. Bot. Upsalienses* 3: 2. 1938.
7. Nordén, Å.: Sporotrichosis. Clinical and laboratory features and a serologic study in experimental animals and humans. — *Acta Path. et Microbiol. Scand. Suppl.* 89. 1951.
8. Ochoa, A. G., Jaber, L. B. & Pacheco, R. S.: Acción estimulante de las sulfonamidas-cobre y de la penicilina sobre el desarrollo de *Cryptococcus neoformans* y *Sporo-*

- trichum Schencki, respectivamente. — La Sociedad Mexikana de Historia Natural 11: 35. 1950.
9. Oyama, T.: Vitamin B und Dermatomyzeten. I. Mitteilung. Einfluss des B-vitamins auf die Dermatomyzeten. — Nagasaki Igakkwai Zasshi 15: 2601. 1937.
 10. Robbins, W. J. & Kavanagh, F.: Vitamin deficiencies of the filamentous fungi. — Bot. Rev. 8: 411. 1942.
 11. Schopfer, W. H.: Les vitamines cristallisées B comme hormones de croissance chez un microorganisme. — Arch. Microbiol. 5: 513. 1934.
 12. — Plants and Vitamins. — Chronica Botanica Company, Waltham. 1949.

Effect of Vernalization and Photoperiodic Treatments on Growth and Development of Crop Plants

IV. Uptake of Nitrogen, Phosphorus, and Potassium by Wheat Plant under varying Photoinductive and Post-Photo-inductive Treatments

By

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In the present paper studies of uptake of nitrogen, phosphorus and potassium by wheat under different photoperiodic treatments are reported. Data for flowering, tiller production, stem elongation, dry matter production and water content have already been given in previous communications of this series (Chinoy and Nanda, Parts I, II, III).

Experimental procedure

A reference may be made to earlier papers of this series for some of the details of experimental procedure.

After the dry weight determinations composite samples of stem, leaves and ears were prepared for the three post-photoinductive treatments separately irrespective of the level of the photoinductive treatment. The samples were dried, powdered so as to pass through a 100-mesh sieve, and analyzed chemically for N, P and K contents. Whole ears as well as grain after removal of husk were analyzed separately. Aliquots were taken from such samples for analysis. Modified Kjeldahl-gunning method (A.O.A.C., 1940) was used for the determination of nitrogen.

For the determination of K and P 3 g. of the powdered material was uniformly spread in a large platinum basin and ignited at first over a low unoxidising flame to drive out fumes, and then on a strong flame. The basin was frequently taken off

the flame and the material stirred by means of a platinum loop. The ignition was continued for about 6 hours to reduce it to constant weight. The resulting ash was dissolved in 5 ml. of 10 per cent HCl and evaporated to dryness. Further 5 ml. of HCl were added. The solution was filtered on a weighed filter paper, thoroughly washed with hot distilled water, made to volume (250 ml.), and kept for P and K analysis after adding a few drops of toluene. The filter paper was dried in an electric oven at 100° C for one hour and weighed to determine the amount of silica present in the sample. Estimation of P_2O_5 was made by the colorimetric method of Fiske and Subbarov (1925). Perchloric acid method was followed for the determination of potassium (A.O.A.C., 1940).

The results are expressed as amount of nitrogen, P_2O_5 , and K_2O , (referred to as N, P and K-contents respectively) per 100 g. of dry weight. The total N, P, and K-contents of individual plant parts and the whole plant are obtained by multiplying the percentage contents with the corresponding dry weights of 100 plants. The absolute rate of uptake of N, P, and K in a given period was derived by multiplying the relative rate of uptake as determined by Verma (1935) by the mean N, P, or K-contents during that period. The mean N, P or K-content was obtained for any given period by dividing the difference in values of two successive samples by the relative rate of uptake during that period.

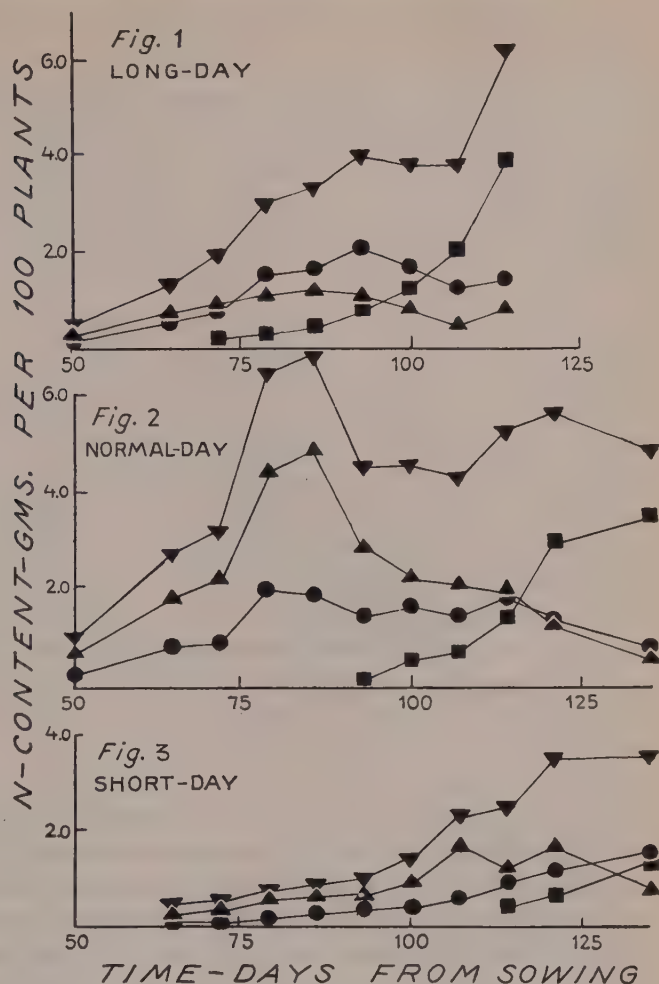
Experimental Findings

Nitrogen Content

N-content as percentage of dry weight is presented in Table 1, for stem, leaf and ear separately. Roots were not analyzed for N, P, and K-contents. The N-content of stem and leaf decreases progressively under all the three

Table 1. *Effect of post photo-inductive treatment on N-content (as percentage of dry weight) at successive developmental stages.*

Time — days from sowing	Post photo-inductive treatment											
	Short day				Normal day				Long day			
	Stem	Leaf	Ear	Grain	Stem	Leaf	Ear	Grain	Stem	Leaf	Ear	Grain
51	—	—	—	—	4.48	4.83	—	—	3.50	3.96	—	—
58	—	—	—	—	—	—	—	—	—	—	—	—
65	3.62	3.69	—	—	2.53	3.81	—	—	1.95	3.93	1.96	—
72	2.31	4.09	—	—	1.90	3.48	—	—	1.79	3.45	1.79	—
79	2.20	3.85	—	—	1.49	3.16	—	—	1.46	3.60	1.36	—
86	2.14	3.37	—	—	0.88	2.88	—	—	1.04	2.92	1.11	—
93	1.82	2.79	—	—	0.65	2.16	1.50	—	1.27	2.93	1.33	—
100	1.53	3.09	—	—	0.68	2.08	1.17	—	1.02	3.00	1.46	2.84
107	0.87	2.98	—	—	0.46	1.56	1.01	—	0.70	2.47	1.57	2.77
114	1.28	3.09	1.50	—	0.51	1.38	1.13	2.81	0.69	2.36	2.15	2.31
121	1.30	2.85	1.65	2.05	0.37	1.07	1.21	2.40	—	—	—	—
128	—	—	—	—	—	—	—	—	—	—	—	—
135	1.37	2.75	1.63	2.40	0.25	0.62	1.35	2.00	—	—	—	—



Figures 1, 2, and 3. Effect of Long-day (Fig. 1), Normal-Day (Fig. 2), and Short-Day (Fig. 3) on total nitrogen content (g. per 100 plants) in different parts of N.P. 52 wheat at successive developmental stages.

photoperiodic treatments. There are, however, considerable differences in the rates of decline. Thus for instance, in stem and leaf of ND plants it is most rapid and consequently the values are much lower than those in LD and SD plants. The decline in N-content of stem and leaf of SD plants is very slow, so much so, that it maintains a higher level at all stages of growth. N-content of ear of LD and ND plants decreases at first with a subsequent rise while those of SD plants registers a continuous fall.

The total N-contents of the whole plant, stem, leaf, and ear for LD, ND, and SD treatments are presented graphically in Figures 1 to 3. The N-content of the whole plant under LD treatment registers a continuous increase. In the stem the N-content progressively increases upto 93 days after which

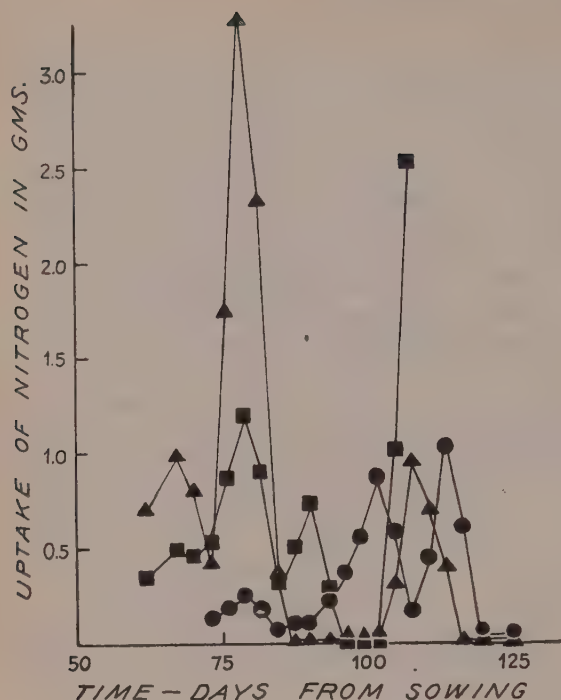


Figure 4. Rate of uptake of nitrogen under the three post-photo-inductive treatments.

■ — ■ = (LD);
 ▲ — ▲ = (ND);
 ● — ● = (SD).

it decreases slowly. In the leaf the maximum is reached after 86 days. The N-content of the ear shows a continuous increase. Under ND treatment the N-content also increases steadily. In stem and leaf this increase continues only till about 86 and 79 days respectively, after which the values maintain a more or less constant value in the case of stem up to 114 days. On the other hand there is a very rapid decline in total N-content of leaf after 86 days. Ears show a continuous increase in their N-content. Although the values of total N-content of the whole plant and leaf are consistently higher in the ND plants compared to LD ones the differences in N-content of the stem under the two treatments are not very marked. The total N-content of SD plants increases very slowly and the values are very much lower compared to those of LD and ND plants. Considering the relative distribution of nitrogen within the plant at different developmental stages, it is seen that the total N-content of the stem surpasses that of leaf of LD plants at a very early stage (72 days after sowing), while the total N-content of leaves remains higher than that of stem up to 114 days in the case of ND plants and upto 128 days in the case of SD plants.

Absolute rates of uptake of nitrogen under the three photoperiodic treat-

ments are shown in Figure 4. The rate increases in the LD and ND plants and reaches a maximum after 72—79 days and decreases rapidly, so much so, that no absorption takes place between the period of 93—107 days. A secondary increase in the absorption rate is noticed during the period between the last two samples. It is worthy of note that the rate of uptake is much higher in ND plants. The rate of uptake of nitrogen is very slow in SD plants till 93 days after which it rises steeply probably due to the termination of the SD treatment.

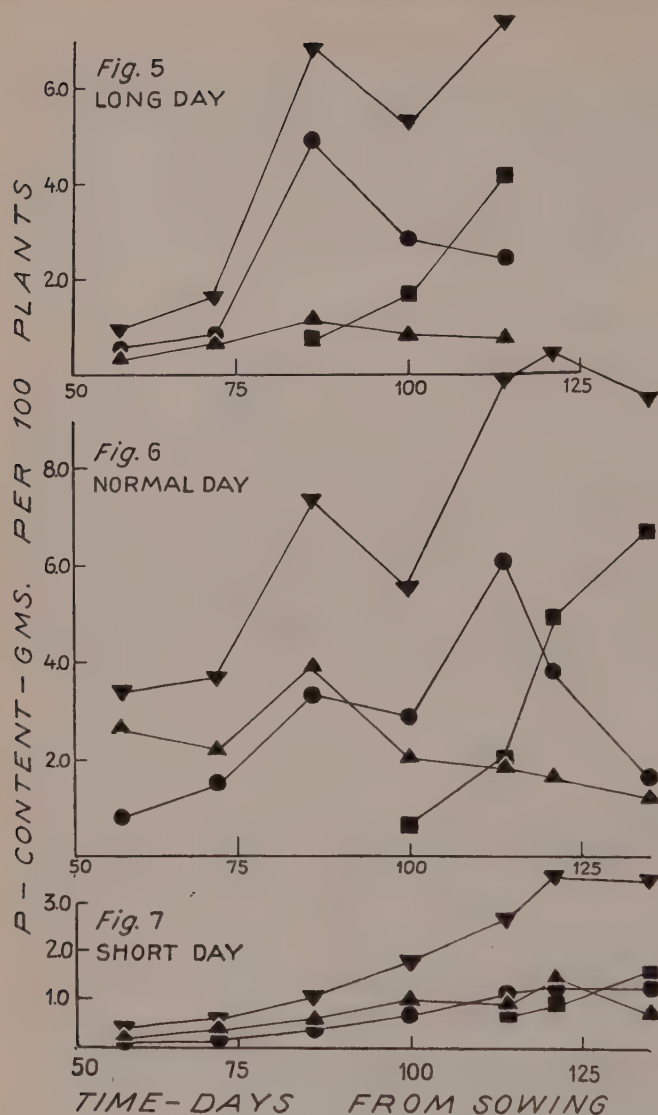
Phosphorus Content

P-content as percentage of dry weight was determined for stem, leaf, and ear separately for the three photoperiodic treatments and the percentage P-content of the whole plant was derived from these values. The results are represented in Table 2. The percentage P-content of stem and leaf of plants under all the three photoperiodic treatments decreases progressively while it increases in the case of ear. The rate of fall, however, differs considerably under the three photoperiodic treatments, being most rapid in ND plants, intermediate in LD plants, and slowest in SD ones. As a consequence of this the percentage P-content in stem and leaf of SD plants is maintained at a higher level than in ND and LD. P-content of ND is the lowest.

Total P-content of the whole plant, stem, leaf and ear are presented graphically in Figures 5 to 7. The total P-content of the whole plant increases with time both in LD and ND plants. The P-content of the stem increases up to 79 days in LD plants and up to 114 days in ND ones after which it gradually declines. The total P-content of leaves on the other hand attains its maximum after 86 days in both cases showing a rapid fall thereafter. It is worthy of note that the total P-content of stem is higher than that of leaf from the very beginning in the case of LD plants while that of the leaf is very much higher in ND plants till about 93 days after which the P-con-

Table 2. *Effect of photoperiodic treatment on P-content (g. per 100 g. dry weight) at successive developmental stages.*

Time — days from sowing	Photoperiodic treatment											
	Short day				Normal day				Long day			
	Stem	Leaf	Ear	Grain	Stem	Leaf	Ear	Grain	Stem	Leaf	Ear	Grain
58	4.03	4.19	—	—	3.98	6.77	—	—	4.43	3.42	—	—
72	3.70	3.74	—	—	3.04	3.51	—	—	1.82	3.26	—	—
86	3.40	3.24	—	—	1.57	2.32	—	—	3.10	2.78	1.76	—
100	2.72	3.33	—	—	1.13	1.87	1.27	—	1.67	2.81	1.97	—
114	1.58	2.56	2.61	—	1.67	1.37	1.52	—	1.14	2.10	2.23	1.98
121	1.40	2.42	2.40	—	1.05	1.30	2.02	1.95	—	—	—	—
135	1.16	2.29	2.06	3.69	0.53	1.21	2.57	2.54	—	—	—	—



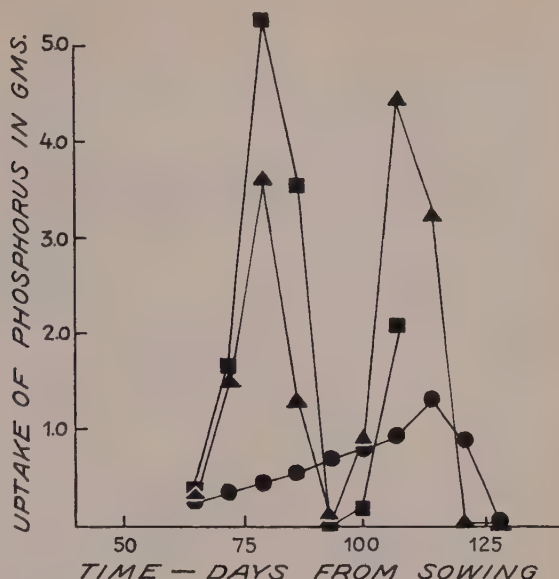
Figures 5, 6, and 7. Effect of Long-Day (Fig. 5), Normal-Day (Fig. 6), and Short-Day (Fig. 7), on total phosphorus content (g. per 100 plants) in different parts of N.P. 52 wheat at successive developmental stages.

tent of the stem overtakes and surpasses that of the leaves. Total P-content of SD plants is very much lower than that of ND and LD plants and the P-content of leaves remains higher than that of the stem till 107 days.

Absolute rates of uptake of P by plants exposed to different photoperiods are presented graphically in Figure 8. The trends followed by the curves are more or less the same as in the case of N-uptake. The two, however, differ

Figure 8. *Rate of uptake of phosphorus under the three post-photo-inductive treatments.*

■ — ■ = (LD);
 ▲ — ▲ = (ND);
 ● — ● = (SD).

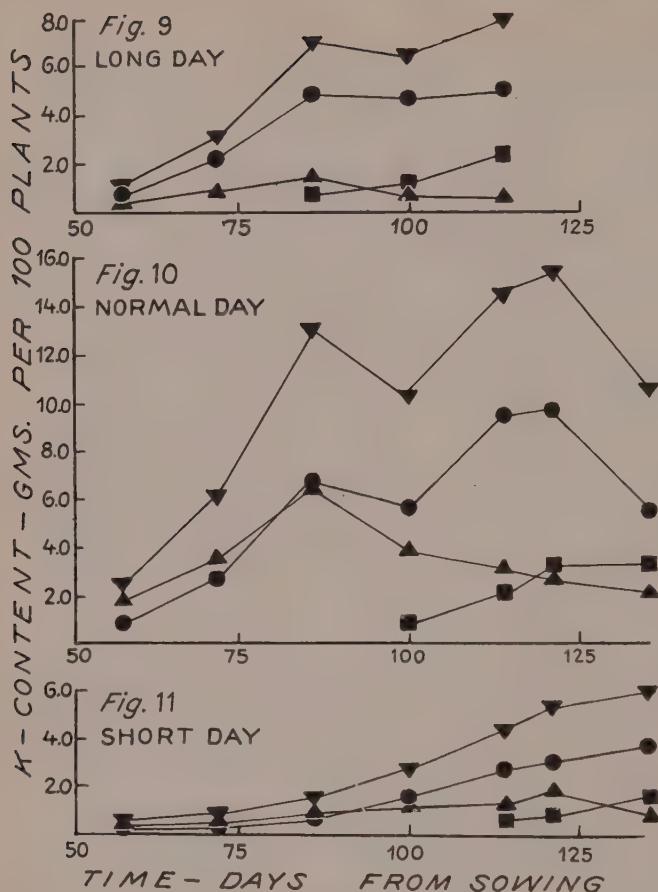


in the fact that whereas the rate of uptake of nitrogen is slightly higher in ND plants that of P is slightly more in plants exposed to LD treatment. As in the case of nitrogen uptake the rate of P-uptake is very slow under SD treatment.

K-content

K-contents as percentages of dry weight of different parts of the plant at successive stages of growth are presented in Table 3. The declining values of percentage K-content also show more or less the same trend as that followed by N and P-contents. The percentage K-content of stem of ND plants rises rapidly to the level of LD ones and then maintains the same trend of fall. The K-content of stem of SD plants remains at a very much higher level without showing any decline up to 100 days after which it gradually falls. The K-content of the leaves of LD plants is the lowest throughout, intermediate in ND and highest in the leaves of SD plants. The K-content of ears under all the three photoperiodic treatments registers a slight fall.

Total K-contents of different parts are given in Figures 9 to 11. The total K-content of the whole plant and stem increases with successive stages. The increase is very rapid at first but slows down later on. The total K-content of the leaves of the LD plants remains at a much lower level from the very beginning than that of the stem; while on the other hand the K-content of



Figures 9, 10, and 11. Effect of Long-Day (Fig. 9), Normal-Day (Fig. 10), and Short-Day (Fig. 11), on total potassium content (g. per 100 plants) in different parts of N.P. 52 wheat at successive developmental stages.

the leaves of ND and SD is higher than that of the stem in the beginning but is soon surpassed by the K-content of the stem (after about 86 days in both cases).

Comparing the distribution of potassium in various plant parts with that of nitrogen, it is interesting to note that while the largest portion of N of the plant migrates to the ear potassium is predominantly present in the stem at the end.

The absolute rates of uptake of K are graphically presented in Figure 12. It is evident that the rate of uptake of potassium resembles closely the rate of nitrogen uptake in all essential details. The absorption is thus much higher under ND treatment compared to LD. The rate is very slow under SD treatment.

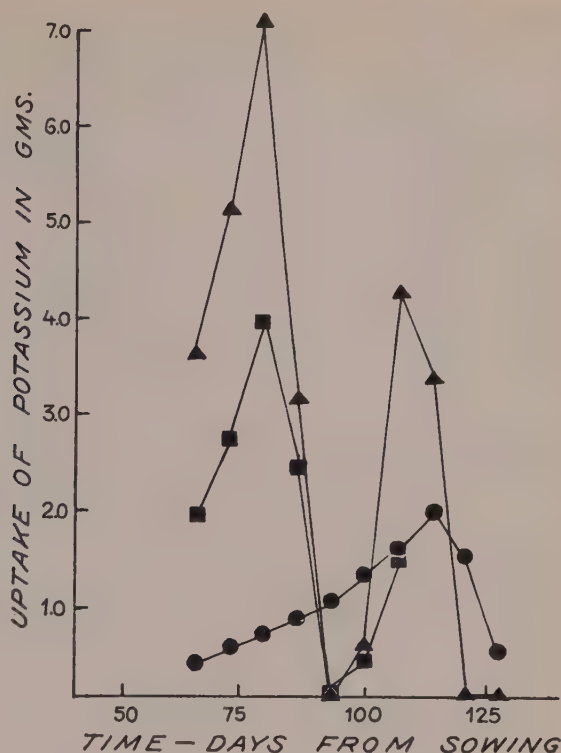


Figure 12. Rate of uptake of potassium under the three post-photo-inductive treatments.

Discussion of Results

Acceleration in stem elongation and retardation in tiller production under the influence of a long photoperiod has already been noted in previous papers of this series. The fact that stem growth is a direct resultant of the developmental process in wheat was further substantiated by growth studies on a large number of wheat varieties (Chinoy, 1949), and their photoperiodic and vernalization responses (Chinoy, 1950). No stem elongation was observed as long as the growing point remained in a vegetative condition. The results of dry matter production, net assimilation rate and water content presented in an earlier communication (Chinoy and Nanda, Pt. III of this series) also lend support to the above conclusions. The ratio of stem weight over leaf dry weight of plants under different photoperiodic treatments (recorded in the above mentioned paper) clearly indicates that early accumulation of dry matter in stem of LD plants is due to acceleration in the developmental process of plants by the long photoperiod. The high net assimilation rate of the LD plants clearly demonstrated that it was not due to any deficiency of carbohydrates that an early break on tillering was imposed.

Table 3. *Effect of post photo-inductive treatment on K-content (as percentage of dry weight) at successive developmental stages.*

Time — days from sowing	Post photo-inductive treatment											
	Short day				Normal day				Long day			
	Stem	Leaf	Ear	Grain	Stem	Leaf	Ear	Grain	Stem	Leaf	Ear	Grain
58	5.80	5.05	—	—	3.22	4.37	—	—	5.49	3.92	—	—
72	5.61	4.84	—	—	5.11	5.27	—	—	4.83	3.63	—	—
86	5.32	4.45	—	—	3.09	3.71	—	—	3.06	3.38	1.60	—
100	5.66	3.78	—	—	2.22	3.49	1.44	—	2.73	2.26	1.35	—
114	3.75	3.12	1.60	—	2.90	2.16	1.36	—	2.37	1.50	1.22	0.93
121	3.20	3.01	1.64	—	2.60	2.08	1.29	0.74	—	—	—	—
135	3.35	2.50	1.89	1.04	1.77	2.04	1.18	0.80	—	—	—	—

The results given in the present communication further substantiate the above findings. The rates of uptake of nitrogen, phosphorus, and potassium by LD plants are sufficiently high to maintain high rates of tiller production or leaf formation. The fact that tiller production and leaf formation are terminated earlier in LD plants cannot, therefore, be ascribed to deficiencies of these elements. It is the mode of partitioning of these minerals between stem and leaf that determines the magnitude of growth in these two plant parts. This is clearly brought out from a study of the ratios of nitrogen, phosphorus, or potassium contents of stem and leaf (Table 4). The ratio reaches a high level (unity) much earlier in LD plants compared to ND or SD ones; and this high level appears to be correlated with the time of flowering in plants under these photoperiodic treatments. It is a direct resultant of the vegetative period of a plant and not due to the length of photoperiod of the treatment is further borne out by the much smaller differences in the leaf-stem dry weight ratios in plants under photoinductive treatments on any given sampling date. Although the daily photoperiod for these treatments is the same in some cases as those for post-photoinductive treatments the differences in vegetative periods are not so pronounced due to the total duration of the treatment being much shorter and hence the differences in ratios for photoinductive treatments are also small.

This earlier transference of nitrogen, phosphorus and potassium to stem in plants flowering earlier under the influence of long photoperiods raises an important issue in mineral nutrition of plants. It would appear as already noted earlier (Chinoy and Nanda, 1949) that the absorption and the distribution of mineral nutrients are controlled by the same regulatory mechanism which controls flowering in a plant. In this connection the differential varietal response to mineral nutrients observed in wheat by Lamb and Salter (1936) and Woodford and MaCalla (1936), in barley by Gregory and Crowther (1928, 1931), in corn by Lynes (1936), and in cotton by Crowther (1936, 1937) and

Table 4. Ratios of nitrogen, phosphorus, and potassium contents of Stem/Leaf in plants exposed to varying post-photoinductive treatments.

Time — days from sowing	Post-photoinductive treatment								
	Short day (SD)			Normal day (ND)			Long day (LD)		
	N	P	K	N	P	K	N	P	K
51	—	—	—	—	—	—	0.70	—	—
58	—	0.39	0.46	—	0.31	0.39	0.71	1.37	1.49
65	0.42	—	—	0.43	—	—	0.72	—	—
72	0.22	0.39	0.48	0.42	0.67	0.75	0.99	1.10	2.56
79	0.33	—	—	0.44	—	—	1.30	—	—
86	0.38	0.64	0.73	0.38	0.84	1.04	1.34	4.23	3.48
93	0.51	—	—	0.47	—	—	1.90	—	—
100	0.39	0.65	1.24	0.74	1.37	1.44	1.89	3.30	6.73
107	0.34	—	—	0.67	—	—	2.37	—	—
114	0.76	1.14	2.21	0.98	3.22	3.20	1.64	3.09	8.83
121	0.71	0.90	1.65	0.99	2.36	3.64	—	—	—
128	—	—	—	—	—	—	—	—	—
135	1.85	1.85	4.99	1.24	1.35	2.67	—	—	—
Vegetative period (Days)	109			99			78		

(N) is the ratio of nitrogen content of stem/leaf.

(P) is the ratio of phosphorus content of stem/leaf.

(K) is the ratio of potassium content of stem/leaf.

Crowther et al. (1935, 1936 & 1937) is worthy of note. Gregory (1937) has given a new orientation to the concept of mineral nutrition in plants by demonstrating the stage of 'Internal Starvation' which marks the maximum in nutrient uptake rate and cessation of exponential growth in leaf area and dry weight increase. 'Internal Starvation' supervenes even under high level of nutrition and appears to bear some relation to the onset of reproductive phase. The partitioning of these nutrients under the influence of photoperiodic treatments bears a strong resemblance to the partitioning that occurs in varieties of wheat differing in their time of flowering (unpublished work).

Parker and Borthwick (1939), Eremenko (1936) and Murneek (1939) have studied the carbohydrate-nitrogen relationship in different plants under the influence of different photoperiods. Parker and Borthwick have shown that the amount of soluble carbohydrates was correlated with the length of the photoperiod and that the total and soluble non-protein nitrogen was more quickly absorbed by plants under 8-hour photoperiod. Eremenko regards C/N ratio as a function of growth and development and not as a causal factor of development. The C/N ratio of plants grown in short photoperiods, which accelerated flowering, increased progressively and the highest total N and protein nitrogen content was also found in plants under short photoperiods. Purvis (1934), and Gregory (1935) have also emphasized that C/N ratio is

Table 5. *C/N, C/P, and C/K ratios in plants exposed to varying photoperiodic treatments.*

Time — days from sowing	Photoperiodic treatment								
	Short day (SD)			Normal day (ND)			Long day (LD)		
	C/N	C/P	C/K	C/N	C/P	C/K	C/N	C/P	C/K
58	—	—	—	28.1	20.3	21.1	25.3	21.0	17.5
65	21.6	20.2	15.3	25.2	19.3	17.9	34.4	36.2	23.1
72	22.2	21.3	15.7	29.6	25.0	15.9	38.0	44.2	22.8
79	24.8	26.0	18.4	37.3	44.8	26.7	46.4	39.1	29.7
86	27.2	24.0	16.6	49.4	45.7	25.9	62.7	30.7	30.2
93	32.5	24.2	16.5	69.5	49.2	27.4	57.2	38.7	35.0
100	33.7	26.4	17.8	79.8	66.1	36.0	65.2	47.4	39.1
107	53.8	55.6	37.0	105.9	60.4	37.5	76.8	47.3	41.7
114	45.5	40.4	26.6	104.2	54.7	38.0	59.6	51.5	48.2
121	44.2	42.7	29.0	114.9	62.3	42.5	—	—	—
128	—	—	—	—	—	—	—	—	—
135	53.1	52.3	32.3	124.9	64.4	58.7	—	—	—

not a causal factor in flowering. Murneek showed that as a result of curtailed growth of plants under short day nitrogen and carbohydrates accumulated in the vegetative parts and was relatively higher at the time of flowering. In other words the C/N ratio was higher in those plants whose development was accelerated by short day.

The relation between the total organic matter and total nitrogen, phosphorus and potassium have been worked out in the present paper and the ratios are presented in Table 5. As the carbohydrate analysis of the plants was not done the total organic matter (C) was determined by subtraction of the quantities of ash and nitrogen from the total dry weight at successive stages of growth. There is a progressive increase in C/N, C/P, and C/K ratios and the higher ratios are attained earliest in LD plants in which the flowering has been considerably accelerated; and this increase in the ratio is progressively delayed with the delay in the time of flowering of ND and SD plants. The ratios do not appear to be constant at the time of flowering under the three photoperiodic treatments. Thus at the time of flowering in LD plants the C/N ratio is about 50, in ND plants it is about 84 and in SD ones about 53. These variations in the ratios at the time of flowering clearly indicate that it cannot be regarded as a causal factor in flowering. As the total organic matter increases even after flowering at a faster rate than N, P or K higher C/N, C/P, and C/K ratios are obtained as the season advances.

Percentage N, P and K contents of leaves and stem (Tables 1, 2, and 3) under different light treatments clearly show that the utilization of these nutrients is impeded both under LD and SD treatments presumably due to different causes. Although the C/N, C/P, and C/K ratios rise much more rapidly

in LD plants the percentage concentrations of N and P of leaves and stem remain at a much higher level than in ND plants, not only at the time of flowering but subsequently for a considerable period of time. In the case of SD plants also percentage concentrations of these nutrients in leaf and stem remain higher than in ND. In the LD plants this higher concentration is probably due to the acceleration in flowering and the consequent check on tiller production and the formation of leaves. In SD plants, on the other hand, this is brought about as a result of the low assimilatory activity (Part III of this series).

Loehwing (1940) has shown that the translocation and distribution of nutrients in the various parts of a plant are brought about as a result of the change in the internal water balance of the plants which is antecedent to flowering. It is worthy of note that similar differences in the water contents of plants under different photoperiodic treatments have been observed as reported earlier (Chinoy and Nanda, Pt. III of this series). Even plants under photoinductive treatment No. 1 (total darkness for the first 14 days), show higher water content during the entire period of growth. In the case of post-photoinductive treatments the water contents of all the parts of LD plants with the exception of leaves declines much more rapidly than in ND and SD plants. The water content of the SD plants is the highest on account of a very slow rate of decline.

Recently Cailajhan and Lukovnikov (1941) have shown that under favourable light conditions development is not affected by varying the available amount of minerals. This fact would also indicate a fortuitous relationship between C/N, C/P, or C/K ratio and flowering. Subsequently, however, Cailajhan (1944, 1945) suggests that a close relationship exists between the time of flowering and the nutritional level in the soil for different plants. On the basis of this work he has classified different plants into three categories, namely, (1) nitronegative plants flowering earlier with little or no nitrogen, (2) nitropositive plants requiring normal or rich supply of nitrogen for early flowering, and (3) nitroneutral plants which do not show any relationship between the level of nitrogen and flowering.

As stated previously there is correlation between mineral nutrition and flowering. Earlier flowering in LD plants causes an earlier transference of these elements from leaf to stem and ear. This appears to be analogous to conditions obtaining in an early flowering variety under normal light conditions. Further the lower rate of uptake of N at the time at which the rate of uptake is the highest in ND suggests that the optimum rate of uptake in LD plants probably reached earlier. As the first sample for nitrogen analysis was taken 45 days after sowing in the present experiment the maximum in the rate is not shown by the curve (Fig. 4 — LD). Differential varietal

response, to which a reference has already been made, is presumably connected with the development of a plant.

At this stage it would be well to hazard a general explanation of the relationship of uptake of nutrients, growth and flowering on the basis of known facts of auxin concentration and transport in plants. It appears to be fairly well established now that auxin is produced in terminal buds as well as in young leaves under the influence of light. Navez (1933) working with *Lupinus* seedlings and van Overbeek (1932, 1933) with *Lepidium* and *Raphanus* seedlings have clearly shown that auxin production takes place only in the presence of light and that in the absence of light the production rapidly falls off. Avery (1935) has also demonstrated in the case of *Nicotiana* that maximum amount of auxin was produced by young leaves only in the presence of light. Thimann and Skoog (1933, 1934) made a thorough study of auxin production in the entire plant of *Vicia Faba* and came to the conclusion that all the young leaves produce auxin in light. As auxin production ceases in the dark but its utilization continues supply of the hormone in different plant parts falls rapidly. Koning (1933) and Avery, Burkholder and Creighton (1937) have demonstrated that the maximum production of auxin in leaves and developing buds coincides with the period of most rapid growth. The effect of light on auxin production is further confirmed by the extraction of auxin by diffusion from the young leaves of *Eleagnus* (van der Weij, 1933). Auxin was also readily obtained by diffusion from the sprouting buds of a number of trees and shrubs, including *Populus*, *Salix*, *Quercus*, and *Aesculus* (Czaja, 1934). Reviewing this work Went and Thimann (1937) came to the conclusion that »light is necessary for the formation of the auxin precursor»; and that the influence of light is not only felt through the photosynthetic production of carbohydrates, but also by the operation of a definite light-sensitive reaction. Earlier Gregory (1928) came to the same conclusion from his studies on the effect of temperature on leaf growth. He postulated that »a master photo-chemical reaction, independent of carbon assimilation, leads to the formation of a substance directly involved in leaf expansion».

In the light of the above evidence it is probable that a greater amount of auxin is produced in the leaves under the influence of long photoperiods (LD). This auxin may, however, diffuse away from light more quickly to darker regions of the plant (root), as first shown by Cholodny (1927) in the case of phototropic curvatures. Went (1928), van Overbeek (1933), and Asana (1936) have demonstrated that greater amount of auxin diffuses out in the agar block placed towards the dark side of the coleoptile compared to the side unilaterally illuminated. This clearly indicated that auxin was transported away from light as postulated by Cholodny (loc. cit.). This accelerated supply of auxin to the root raises its concentration above the physiological limit thus putting a check on root growth. There is ample evidence to show that retardation in root growth occurs in higher concentrations of auxins (Nielsen, 1930; Navez, 1933; Boysen Jensen, 1933; Keeble, Nelson and Snow, 1930; Kögl, Haagen Smit, and Erxleben, 1934; Lane, 1936; Meesters, 1936 and Thimann, 1935). As a consequence of this check on root

growth imposed by higher concentration of auxin the rate of uptake of the nutrient registers an earlier fall in LD plants, thus causing »internal starvation» as postulated by Gregory (1937).

This reduction in the uptake of nutrients is, however, not responsible for the early termination of tiller production in LD plants as pointed out at the beginning of this discussion. Maintenance of appreciably higher percentage concentrations of nitrogen and phosphorus in leaves of LD plants (Tables 1 and 2) also lends support to this view. In spite of the earlier transference of these nutrients to stem and ear in these plants the concentration in leaf remains higher. It is, therefore, clear that suppression of tillering is due to the reduction in the number of leaf primordia in the first instance and also due to the arrest of growth of tiller initials arising in the axil of leaves. Both are the resultant of inhibitory concentration of auxin in the growing point. Ample evidence is at hand to show that such inhibitory action is exerted by auxin on bud development (Thimann and Skoog, 1933, 1934; Avery, 1935; Goodwin, 1937; Thimann, 1935; Skoog and Thimann, 1934; Hitchcock, 1935; Muller, 1935). At this stage it is worthy of note that the much-branched habit of *Aster multiflorus* was found to be correlated with very small auxin production by the buds, while on the other hand, *A. novae-Angliae* produced considerably more and as a consequence of which remained in almost an unbranched condition (Delisle, 1937). van Overbeek (1935) has shown in the case of *nana* form of *Zea Mays* that dwarfing is due to a reduction in the amount of auxin present. Taking this into consideration Went and Thimann (1937) observe that the much branching habit of many dwarfs, such as those of *Pisum*, *Vicia*, and *Zea* may be due to reduction in the amount of auxin. The inhibiting action of auxin on tillering is probably exerted through its influence on the translocation of nutrients. Higher concentration of nitrogen and phosphorus in the leaves of LD plants as well as its earlier transference to stem and ear would indicate that these nutrients tend to accumulate at the centres of auxin production, *viz.*, leaf and the actively growing terminal bud. Went (1936) suggested such a mechanism of inhibition of bud development in etiolated *Pisum* seedlings. Results of potassium content are most interesting from the point of view of transport of materials. Unlike nitrogen and phosphorus the percentage concentration of potassium of the leaves of LD plants falls much more rapidly than that in the leaves of ND and SD plants (Table 3). The ratio of K-content of stem/leaf also registers a sharp increase (Table 4) compared to those of N and P in LD plants and is also much higher in these plants in comparison to ratios of K-content of stem/leaf in ND and SD plants. Another important point of difference is the final K-content of stem. Where as the largest portions of nitrogen and phosphorus are transferred to ear and grain at the end, potas-

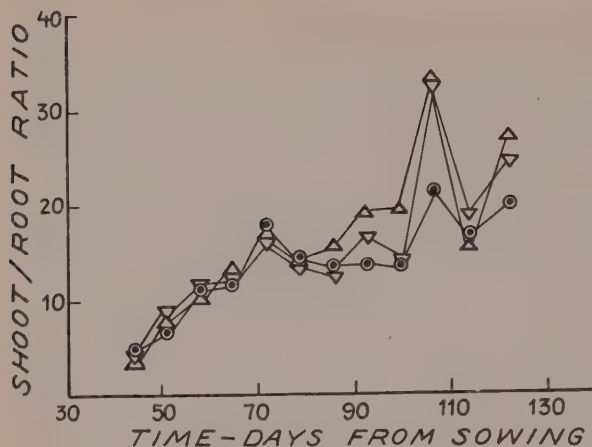


Figure 13. Shoot-Root ratio under three photoinductive treatments.

○—○ = No. 1
 △—△ = No. 3
 ▽—▽ = No. 5

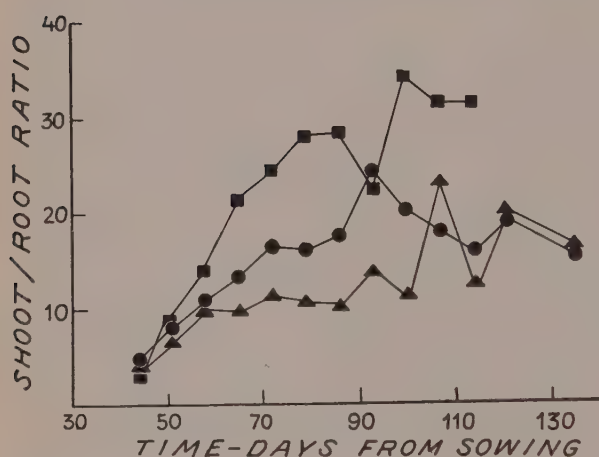


Figure 14. Shoot-Root ratio under three post-photoinductive treatments.

■—■ = (LD)
 ▲—▲ = (ND)
 ●—● = (SD)

sium is present predominantly in the stem under all the three light treatments (Figures 9, 10, 11). All these facts suggest that potassium is not only more intimately connected with flowering than N and P, but is also playing some vital role in the transport of nutritional and hormonal factors necessary for growth and flowering. A reference may be made here to the work of Gregory (1937) and his collaborators (Mathur, 1934; Verma, 1935) on the effects of mineral deficiencies in barley on growth and flowering. A high potassium deficiency caused excessive tillering and delayed flowering as well as retarded grain formation. Potassium deficiency caused considerable accumulation of amino acids and as a consequence of which the rate of respiration was found to be supernormal. Excessive tillering occurred in spite of the fact that the rate of carbon assimilation was considerably reduced

Table 6. *Leaf/Root weight ratio in plants under photoinductive and post-photoinductive treatments.*

Time — days from sowing	Photoinductive treatment			Post-photoinductive treatment		
	No. 1	No. 3	No. 5	Short day	Normal day	Long day
51	2.7	2.0	2.6	2.6	2.7	3.7
58	3.0	3.9	4.0	3.0	4.0	3.5
65	4.2	4.8	4.8	3.6	4.5	6.1
72	3.8	3.5	5.5	4.9	5.2	6.1
79	3.4	3.7	9.8	6.8	3.1	3.5
86	2.2	3.1	3.4	3.9	2.7	3.0
93	2.5	3.0	2.3	6.8	2.4	2.5
100	2.4	3.4	1.9	6.5	2.5	1.6
107	2.1	2.7	1.2	6.1	1.5	2.2
114	2.9	3.8	1.1	6.0	1.8	1.3
121	1.8	2.8	1.7	2.2	2.1	2.4
128	2.3	3.1	2.2	2.7	2.5	—
135	—	—	—	1.3	1.9	—

under potassium deficiency (Gregory, 1937; Jones, 1925; Gregory and Sen, 1937). Auxins are inactivated by enzymes (Thimann, 1934; van Overbeek, 1935; Bonner and Thimann, 1935; Fiedler, 1936; Larsen, 1936; and Kornmann, 1936). It is, therefore, most probable that higher oxidative activity under potassium deficiency found by Gregory and Sen (1937) and Mathur (1934) may have reduced the concentration of auxin to a sufficiently low level so as to cause excessive tillering in potassium deficient plants. The transport of auxin may also have been impeded on account of low potassium level in the plant.

The shoot-root ratios which are presented in Figures 13 and 14 appear to be similar for the photoinductive treatment Nos. 3 and 5 upto 93 days after which they show a definite increase over No. 1 treatment, probably on account of earlier flowering and grain formation. In post-photoinductive treatment the ratio shows an early rise in the case of LD plants again on account of early stem elongation followed by flowering and grain formation. A similar rise in the ratio is obtained in ND plants after 99 days which synchronizes with its time of flowering. That this increment in the ratio is due to the formation of stem and ear and grain development, and not on account of tiller production or leaf growth is clearly seen from the data of leaf-root weight ratios given in Table 6. Obviously, therefore, the nutrients required for the development of stem, ear and grain were absorbed by the plant earlier in its life. This would mean that a considerable part of its dry weight was added during the period in which root growth had almost stopped and the uptake of nutrients was very low (Figs. 4, 8, 12).

It has been observed in the foregoing paragraphs that the early stoppage of growth in roots and tillers is due to a high concentration of auxin in the

plants under LD treatment. This is further substantiated by the fact that tillering and root growth are resumed in LD plants after the grain formation has reached a certain stage (half-ripe stage). Considerable evidence is at hand to show that a developing grain accumulates auxin in large quantities (Cholodny, 1935; Schander, 1934; Thimann and Skoog, 1940; Haagen-Smit, Leech and Bergren, 1942; Hatcher and Gregory, 1941; Hatcher, 1943; and Avery, Berger and Shalucha, 1941, 1942). In the present work resumption of root growth in LD plants after 100 days with a consequent secondary rise in rates of uptake of nutrients and formation of a secondary crop of tillers is presumably due to the lowering of auxin concentration which is conducive to tiller formation. Such a phenomenon of resumption of tiller production has also been observed in the case of some early varieties of wheat under favourable environmental conditions.

On the other hand restriction in root growth and tiller production in SD plants is not due to higher auxin concentration, but is due to the limitation imposed on carbon assimilation under a very short period of illumination. This becomes quite clear from the rapid increase in growth and uptake of minerals after 100 days when the short day treatment is terminated.

Summary

1. Nitrogen, phosphorus, and potassium contents of different plant parts of one variety of wheat (*Triticum vulgare*- N.P. 52) under different photo-periodic treatments were determined at successive stages of growth. The rates of uptake of these nutrients were also determined from the dry weight data and N, P and K-contents.

2. Percentage nitrogen and phosphorus contents of stem and leaf decrease progressively. This decrease is more rapid in ND plants compared with LD ones. In SD plants the fall is very gradual and therefore, the values remain higher under this treatment. The potassium contents of LD and ND plants do not vary significantly. That of leaves, however, is lower in LD plants. The K-content of ear decreases slightly.

3. The total nitrogen, phosphorus, and potassium-contents of the whole plant as well as the leaf are higher in all cases in ND plants compared to LD treatment. The differences in total N, P, and K-contents of stem, on the other hand, do not appear to be marked. Levels of the three nutrients are very low in SD plants.

4. Rates of uptake of nitrogen and potassium are higher in ND plants, while on the other hand the rate of uptake of phosphorus is greater in LD plants. Rates of uptake of all the three nutrients are the slowest in SD plants.

5. Comparing the distribution of the three nutrients at the end of the growing season it is seen that whereas the largest proportion of nitrogen and phosphorus is found in the ear, potassium is predominantly present in the stem.

6. From a study of the ratios of N, P and K-contents of stem and leaf it has been concluded that the mode of partitioning of these minerals between the stem and the leaf determines the magnitude of growth in these two plant parts. Earlier flowering in LD plants causes an earlier transference of these elements from leaf to stem and ear as is evident from an earlier increase to unity of the nutrient ratios in these plants.

7. Effects of photoperiodic treatments on growth, development, and mineral nutrition of wheat have been discussed on the basis of the influence of light on the production and transport of auxins. It is suggested that the regulatory mechanism which puts an early check on tiller production and leaf dry weight increase in wheat under the influence of long photoperiod is similar to that found in an early variety when compared to a late flowering one. This regulatory mechanism also controls mineral uptake and its distribution to various plant parts.

References

- A.O.A.C. Official and tentative methods of analysis. Association of Official Agricultural Chemists. 1940.
- Asana, R. D.: On the relation between the distribution of Auxin in the tip of the *Avena* coleoptile and the first negative phototropic curvature. — Ann. Bot. N.S. 2: 955. 1938.
- Avery, G. S.: Differential distribution of phytohormone in the developing leaf of *Nicotiana* and its relation to polarised growth. — Bull. Torey Bot. Club 62: 313. 1935.
- Berger, J. & Shalucha, B.: The total extraction of free auxin and auxin precursor from plant tissue. — Amer. J. Bot. 28: 596. 1941.
- — Auxin content of maize kernels during ontogeny, from plants of varying heterotic vigour. — Amer. J. Bot. 29: 765. 1942.
- Burkholder, P. R., & Creighton, H. B.: Production and distribution of growth hormones in shoots of *Aesculus* and *Malus* and its probable role in stimulating cambial activity. — Amer. J. Bot. 24: 51. 1937.
- Bonner, J. & Thimann, K. V.: Studies on the growth hormone of plants. VII. The fate of growth substance in the plant and the nature of the growth process. — J. Gen. Physiol. 18: 649. 1935.
- Boysen-Jensen, P.: Über die durch einseitige Lichtwirkung hervorgerufene transversale Leitung des Wuchsstoffes in der *Avena* Koeoptile. — Planta 19: 335. 1933.
- Cailajhan, M. H. & Lukovnikov, E. K.: Development of plants growing under different light conditions as affected by mineral nutrition. — C. R. (Doklady) Acad. Sci. U.R.S.S. 32: 152. 1941. (Quoted from Herb. Abst. Oct. 1942).
- Cailajhan, M. H.: Contribution to the analysis of the theory of flowering of plants. — C. R. (Doklady) Acad. Sci. U.R.S.S. 44: 348. 1944. (Rev. in Herb. Abst. June 1945).
- On the correlation between the reaction of flowering to nitrogenous food and photo-

- periodic reaction of the plant. — C. R. (Doklady) Acad. Sci. U.R.S.S. 48: 360. 1945.
(Quoted from Herb. Abst. Feb. 1946).
- Chinoy, J. J.: Correlation between growth and development. — *Nature* (London) 164: 879. 1949.
- Effect of vernalization and photoperiodic treatments on grain development in wheat. — *Curr. Sci.* 18: 414. 1949 a.
 - Effect of vernalization and photoperiodic treatments on growth and development of wheat. — *Nature* (London), 165: 882. 1950.
 - & Nanda, K. K.: Photoperiodic treatment and nitrogen uptake in wheat. — *Curr. Sci.* 19: 24. 1950.
 - — Effect of vernalization and photoperiodic treatments on growth and development of crop plants. Part I. Varietal differences in flowering of wheat and its correlation with spike growth, under varying photoinductive and post-photoinductive treatments. — *Physiol. Plant.* 4: 209. 1951.
 - — Effect of vernalization and photoperiodic treatments on growth and development of crop plants. Part II. Varietal differences in stem elongation and tillering of wheat and their correlation with flowering under varying photoinductive and post-photoinductive treatments. — *Ibid.* 4: 427. 1951.
 - — Effect of vernalization and photoperiodic treatments on growth and development of crop plants. Part III. Rate of dry matter production, net assimilation rate, and water content of wheat under varying photoinductive and post-photoinductive treatments. — *Ibid.* 4: 575. 1951.
- Cholodny, N.: Wuchshormone und Tropismen bei den Pflanzen. — *Biol. Zentralbl.* 47: 604. 1927.
- Investigations on the growth hormone of plants in the U.S.S.R. — *Herb. Rev.* 3: 210. 1935.
- Crowther, F.: Experiments in Egypt on the interaction of factors in crop growth. 7. The influence of manuring on the development of the cotton crop. — *Roy. Agri. Soc., Egypt, Bull. No. 31* (Techn. Sec.). 1937.
- & Mahmoud, A.: Experiments in Egypt on the interaction of factors in crop growth. I. A Preliminary investigation of variety, spacing, nitrogen and water supply, with reference to yields of cotton. — *Roy. Agri. Soc., Egypt, Bull. No. 22* (Techn. Sec.). 1935.
 - — Experiments in Egypt on the interaction of factors in crop growth. 3. The effects of variety, spacing, nitrogen and water supply on the development of the cotton plant and the rate of its absorption of nitrogenous fertilizers. — *Roy. Agri. Soc., Egypt, Bull. No. 25* (Techn. Sec.). 1936.
 - Tomforde, A. & Mahmoud, A.: Experiments in Egypt on the interaction of factors in crop growth. 4. Nitrogenous and phosphatic manuring of cotton and their relation to variety and spacing. — *Roy. Agri. Soc., Egypt, Bull. No. 26* (Techn. Sec.). 1936.
 - — — Experiments in Egypt on the interaction of factors in crop growth. 8. Manuring of cotton in Egypt. — *Roy. Agri. Soc., Egypt, Bull. No. 32* (Techn. Sec.). 1937.
- Czaja, A. Th.: Der Nachweis des Wuchsstoffes bei Holzpflanzen. — *Ber. d. bot. Ges.* 52: 267. 1934.
- Delisle, A. F.: The influence of auxin on secondary branching in two species of *Aster*. — *Amer. J. Bot.* 24: 159. 1937.
- Eremenko, V. T.: Zbirn. Prac. (Rob.) Agrofiziol. 2: 3. 1936. (Quoted from Whyte, 1946, pp. 185).

- Fiedler, H.: Entwicklungs- und reiz-physiologische Untersuchungen an Kulturen isolierter Wurzelspitzen. — Z. f. Bot. 30: 385. 1936.
- Fiske, C. H. & Subbarow, Y. P.: The colorimetric determination of phosphorus. — J. Biol. Chem. 66: 375. 1925.
- Goodwin, R. H.: The role of auxin in leaf development in *Solidago* species. — Amer. J. Bot. 24: 43. 1937.
- Gregory, F. G.: Effect of climatic conditions on the growth of barley. — Ann. Bot. 40: 1. 1926.
- Photoperiodicity in cereals. — Zesde Internl. Bot. Congr. 2: 18. 1935.
 - & Crowther, F.: A physiological study of varietal differences in plants. I. A survey of the comparative yields of barley varieties with different manurings. — Ann. Bot. 42: 757. 1928.
 - — A physiological study of varietal differences in Plants. II. Further evidence for the differential response in yield of barley varieties to mineral deficiencies. — Ann. Bot. 45: 579. 1931.
 - & Sen, P. K.: Physiological studies in plant nutrition. VI. The relation of respiration rate to the carbohydrate and nitrogen metabolism of barley leaf as determined by nitrogen and potassium deficiency. — Ann. Bot. N.S. 1: 521. 1937.
- Haagen-Smit, A. J., Leach, W. D. & Bergren, W. R.: The estimation, isolation and identification of auxins in plant materials. — Amer. J. Bot. 29: 500. 1942.
- Hatcher, E. S. J.: Auxin production during development of the grain in the cereals. — Nature (Lond.) 151: 278. 1943.
- & Gregory, F. G.: Auxin production during the development of the grain in cereals. — Nature (Lond.), 148: 623. 1941.
- Hitchcock, A. E.: Indole-3-n-propionic acid as a growth hormone and the quantitative measurement. — Contrib. Boyce Thompson Inst. 7: 87. 1935.
- Jones, R. L. & Pember, F. R.: The fertilizer nutrients required by barley, wheat and oats as shown by both soil and water cultures. — Soil Sci 19: 169. 1925.
- Keeble, F., Nelson, M. G. & Snow, R.: A wound substance retarding growth in roots. — New Phytol. 29: 289. 1930.
- Kögl, F., Haagen-Smit, A. J. & Erxleben, H.: Über den Einfluss der Auxine auf das Wurzelwachstum und über chemische natur des Auxins der Graskoleoptilen. XII. Mitteilung. — Z. physiol. Chem. 228: 104. 1934.
- Koning, H. C.: Het winden der Slingerplanten (Quoted from Went and Thimann, 1937) 1933.
- Kornmann, P.: Die Aufhebung der Wuchsstoffwirkung durch lebende Pflanzenteile. — Ber. d. bot. Ges. 53: 523. 1935.
- Lamb, C. A. & Salter, R. M.: Response of wheat varieties to different fertility levels. — J. Agri. Res. 53: 129. 1936.
- Lane, R. H.: The inhibition of roots by growth hormone. — Amer. J. Bot. 23: 532. 1936.
- Larsen, P.: Über einen wuchsstoffinaktivierenden Stoff aus *Phaseolus-Keimpflanzen*. — Planta, 25: 311. 1936.
- Lynes, A. S.: Varietal differences in the phosphorus feeding capacity of plants. — Plant Physiol. 11: 665. 1936.
- Loehwing, W. F.: Mineral nutrients in relation to flower development. — Science 92: 517. 1940.
- Mathur, R. N.: Inter-relations of nitrogen and potassium in the growth of barley. — Thesis for Ph. D., London University. 1933.

- Meesters, A.: The influence of heteroauxin on the growth of root hairs and roots of *Agrostemma githago* L. — Proc. Kon. Akad. Wetensch. Amsterdam 39: 91. 1936.
- Müller, A. M.: Über den Einfluss von Wuchsstoff auf das Austreiben der Seitenknospen und auf die Wurzelbildung. — Jahrb. wiss. Bot. 81: 497. 1935.
- Murneek, A. E.: Physiological factors in reproduction of plants. — Growth 3: 295. 1939.
- Navez, A. E.: Geo-growth reaction of roots of *Lupinus*. — Bot. Gaz. 24: 616. 1933.
- Nielsen, N.: Untersuchungen über einen neuen Wachstumsregulierenden Stoff: Rhizopin. — Jahrb. wiss. Bot. 73: 125. 1930.
- van Overbeek, J.: An analysis of phototropism in dicotyledons. — Proc. Kon. Akad. Wetensch. Amsterdam. 35: 1325. 1932.
- Wuchsstoff, Lichtwachstumsreaktion und Phototropismus bei *Raphanus*. — Rec. trav. bot. neerl. 30: 537. 1933.
- The growth hormone and the dwarf type of growth in corn. — Proc. Nat. Acad. Sci. 21: 292. 1935.
- Parker, M. W. & Borthwick, H. A.: Effect of photoperiod on development and metabolism of the Bilox soy bean. — Bot. Gaz. 100: 651. 1939.
- Purvis, O. N.: An analysis of the influence of temperature during germination on the subsequent development of certain winter cereals and its relation to the effect of length of day. — Ann. Bot. 48: 919. 1934.
- Skoog, F. & Thimann, K. V.: Further experiments on the inhibition of the development of lateral buds by growth hormone. — Proc. Nat. Acad. Sci. 20: 480. 1934.
- Thimann, K. V.: Studies on the growth hormone of plants. VI. The distribution of growth substance in plant tissues. — J. Gen. Physiol. 18: 23. 1934.
- Growth substances in plants. — Ann. Rev. Biochem. 4: 445. 1935.
- & Skoog, F.: Studies on the growth hormone of plants. III. The inhibitory action of the growth substance on bud development. — Proc. Nat. Acad. Sci. 19: 714. 1933.
- — On the inhibition of bud development and other functions of growth substance in *Vicia faba*. — Proc. Roy. Soc. B 114: 317. 1934.
- — The extraction of auxin from plant tissues. — Amer. J. Bot. 27: 951. 1940.
- Verma S. S.: Interaction of phosphorus and potassium in the growth of barley. — Thesis for Ph. D., London University. 1935.
- van der Weij, H. G.: Über das Vorkommen von Wuchsstoff bei Meeresalgen. — Pubbl. Staz. Zool. Napoli, 13: 172. 1933.
- Went, F. W.: Wuchsstoff und Wachstum. — Rec. trav. bot. neerl. 25: 1. 1928.
- The growth hormone in plants. — Proc. 25th Ann. Celebration Univ. Calif. p. 223. 1936. (Went and Thimann, 1937).
- & Thimann, K. V.: The Phytohormones. — The Macmillan Co., New York 1937.
- Whyte, R. O.: Crop Production and Environment. — Faber and Faber, London 1946.
- Woodford, E. K. & MacCalla, A. G.: The absorption of nutrients by two varieties of wheat grown on the black and gray soils of Alberta. — Can. J. Res. 14: 245. 1936.

Pine Tissue Cultures

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It is common knowledge that the vegetative propagation of conifers is difficult. One approach to the problem is the study of the nutritional requirements of coniferous tissue by in vitro culture techniques. Although Loo and Wang (1943) have obtained limited growth of *Pinus Yunnanensis* and *Keteleeria davidiana* embryos, and Slankis (1950, '51) has succeeded in culturing excised root tips of *Pinus silvestris* on a mineral medium supplemented by sucrose and growth factors, the only reported instance of cultivation of coniferous callus tissue in vitro is the work of Ball (1950) on *Sequoia sempervirens*. Using the rapidly proliferating redwood burl as starting material for his cultures, Ball found that the organic requirements of *Sequoia sempervirens* can be met by sucrose and auxin. Wetmore and Morel (private communication) have been able to grow *Pinus strobus* callus on a medium supplemented by sucrose, auxin, and a mixture of B vitamins. This paper describes a method for obtaining tissue cultures of *Pinus banksiana* and a medium for maintaining their continuous growth in subcultures. Callus of *P. strobus* has also been obtained and grown by the same techniques.

Procedure for obtaining callus: Seeds of *P. banksiana* were sterilized by immersion for ten minutes in a calcium hypochlorite solution (Wilson, 1915) containing four per cent available chlorine, followed by soaking for one half to two hours in a three per cent hydrogen peroxide solution. The seeds were then placed in Petri dishes containing three layers of filter paper and five ml. of sterile water. Ten to fifteen seeds were placed in each dish. To reduce evaporation and contamination the dishes were taped around the edge with masking tape. After one to two weeks at room temperature, when the seed-

lings were about four centimeters long, they were cut in half and planted on fifty ml. agar medium in one hundred and twenty five ml. Erlenmeyer flasks. To induce *P. strobus* seeds to germinate, the Petri dishes containing the sterilized seeds were kept at 7° C for six weeks before they were moved to room temperature.

All attempts to obtain pine tissue cultures on mineral sucrose media, even with the addition of various known vitamins, coconut milk (cold filtered or autoclaved), yeast extract, honey and peptone, corn steep, and cow's milk (cold filtered or autoclaved) failed. Considerable callus formation was obtained, however, on a medium supplemented with malt extract and naphthaleneacetic acid. The most satisfactory medium found so far for the production of callus has the following composition per liter of aqueous solution:

Ca(NO ₃) ₂ · 4H ₂ O	500	mg	choline chloride	0.5	mg
MgSO ₄ · 7H ₂ O	125	mg	thiamine	0.2	mg
KNO ₃	125	mg	naphthaleneacetic acid	1.0	mg
KH ₂ PO ₄ (Gautheret 1942)	125	mg	cysteine hydrochloride	10.0	mg
Berthelot solution (1934) (Beryl-			sucrose	20	gm
lium omitted)	0.5	ml	agar	10	gm
			malt solution	100	ml

The malt solution was prepared by adding twenty grams of coarsely ground malted barley seed to one hundred ml of double distilled water; the mixture was kept at 37° C for one hour and then at 55° C for a second hour. Coarse solids were removed by straining through cheesecloth, and fine matter either by centrifugation at 1000 G for half an hour or by three filtrations through successively finer filter papers. The resulting clear yellow liquid was sterilized by filtration through a Seitz filter. The medium, complete but for the malt, was autoclaved at fifteen pounds pressure for fifteen minutes; after it had cooled to 45° C, the malt solution was added. Seedlings planted on this medium showed little callusing during the first month, but during the next two months up to thirty per cent of the seedlings produced vigorously growing callus.

Maintenance of callus growth: Although autoclaved malt extract induced some callus on *P. banksiana* seedlings, it did not support continuous growth of the subcultures. Therefore a variety of media have been tried for subculturing the callus. Only on media supplemented with either unautoclaved malt solution (10 per cent) or unautoclaved pine seed extract did the subcultures continue to grow. (The pine seed extract was made from thirty three grams of *P. banksiana* seed soaked in one hundred ml. double distilled water for thirteen hours at 7° C. The water was discarded, and the seeds were ground in a Waring blender with one hundred and sixty five ml. of fresh water per thirty three grams of seed. The solution was clarified and

filtered exactly as the malt solution.) *P. banksiana* callus has been carried through seven passages and is now growing well in the eighth transfer on these media. Cultures of *P. banksiana* kept in dim light at 22° C on a medium containing 0.1 mg/liter naphthaleneacetic acid increase their size about ten-fold within a month when the explants are of the order of thirty mm³; but faster growth rates have been observed frequently. Callus that is not subcultured after one month continues to grow but tends to brown in the older parts. *P. strobus* callus appears to grow somewhat faster, and is now in the fourth transfer.

Morel and Wetmore (private communication) have obtained callus from the cambium of mature *P. strobus* trees that is now in the third year of culture. Stem segments were planted on a medium containing Knop's solution, Berthelot's solution, a mixture of B vitamins, dextrose, $5 \cdot 10^{-8}$ naphthaleneacetic acid, and ascorbic acid (Wetmore and Morel, 1949). Several days later the pieces were transplanted onto a similar medium but without ascorbic acid. During the last year their cultures have been kept on a medium in which coconut milk (15 per cent) has been substituted for the B vitamin mixture and sucrose (2 per cent) for the dextrose. *P. strobus* callus similarly obtained from stem segments, but cultured on the malt-containing medium described above, is now growing in the first subculture.

A growth factor required by P. banksiana callus: Since autoclaved malt solution is ineffective for maintenance of callus growth, and combinations of autoclaved and unautoclaved malt produce approximately the same effect as unautoclaved malt alone, it is apparent that the essential factor(s) required by *P. banksiana* callus is heat labile, and that the ineffectiveness of autoclaved malt solution is not due to toxic substances. Both autoclaved and unautoclaved coconut milk were ineffective in inducing callus formation in *P. banksiana* seedlings in the present study, while malt solution was effective. Therefore, the required factor cannot be identical with the heat stable coconut factor of Caplin and Steward (1948), but possibly it is identical with the heat labile factor in barley endosperm used by Ziebur and Brink (1951) for the culture of barley embryos.

A study of the factor(s) in malt responsible for growth of *P. banksiana* callus is in progress.

Summary

A method and media for obtaining callus and maintaining growth of tissue cultures of *Pinus banksiana* and *P. strobus* are described. A heat labile

factor present in malt and pine seed appears to be essential for the growth of *P. banksiana* cultures.

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References

- Ball, E.: Differentiation in a callus culture of *Sequoia sempervirens*. — Growth 14: 295. 1950.
- Berthelot, A.: Nouvelle remarques d'ordre chimique sur le choix des milieux de culture naturels et sur la maniere de formuler les milieux synthetiques. — Bull. Soc. chim. biol. 16: 1553. 1934.
- Caplin, S. M. and Steward, F. C.: Effect of coconut milk on the growth of explants from carrot root. — Science 108: 655. 1948.
- Gautheret, R. J.: Manuel de technique de culture des tissus vegetaux. Page 51. — Masson et Cie., Paris, 1942.
- Loo, S. W., and Wang, F. H.: The culture of young conifer embryos in vitro. — Science 98: 544. 1943.
- Slankis, V.: The effect of naphthaleneacetic acid on dichotomous branching of isolated roots of *Pinus silvestris*. — Physiol. Plant. 3: 40. 1950.
- Slankis, V.: Über den Einfluss von β -Indolylessigsäure und anderen Wuchsstoffen auf das Wachstum von Kiefernurzeln. I. — Symb. Bot. Upsalienses II: (3) 1. 1951.
- Wetmore, R. H. and Morel, G.: Polyphenol oxidase as a problem in tissue culture and auxin diffusion of horsetails and ferns. — Am. J. Bot. 36: 830. 1949.
- Wilson, J. K.: Calcium hypochlorite as a seed sterilizer. — Am. J. Bot. 2: 420. 1915.
- Ziebur, N. K. and Brink, R. A.: The stimulative effect of *Hordeum* endosperm on the growth of immature plant embryos in vitro. — Am. J. Bot. 38: 253. 1951.

Preliminary Studies on the Synthesis of Polysaccharides in the Algae. II. A Polysaccharide Variant of *Oscillatoria Princeps*

By

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The intimate relationship between genes and those enzymes involved in polysaccharide synthesis and the carbohydrate cycle in plants was investigated by Brink (2). His work with maize showed that variations occurred in the type of polysaccharide present in maize pollen, and that this variation was linked to the texture of the pollen grain. A heterozygous *waxy* plant produced two types of pollen, one of which stained blue with iodine, and the other of which stained red-brown. A normal plant produces *non-waxy* pollen which stains blue with iodine.

This was interpreted by Brink to mean that the variation in polysaccharide was due to a difference in the enzymes (in this case, the amylases) responsible for its synthesis, and that this difference in enzymes was due to the presence of the gene for *waxy*.

In the blue-green alga, *Oscillatoria princeps*, the polysaccharide synthesized has a violet iodine color which shows maximum absorption at 550 m μ . It is soluble in cold water and resembles animal glycogen in many of its properties (3).

Methods and Results

A transfer culture of *Oscillatoria princeps*, growing on Chu No. 10, as modified by Gerloff (5), when tested by the extract method described (3), synthesized a white polysaccharide from hexose monophosphate (Schwarz Labs.,

Table 1. *Iodine absorption spectra of polysaccharide synthesized by a mutant culture of Oscillatoria.*

Time	Colour	Max absorption m μ
10 minutes	violet	560
20 »	violet-blue	600
30 »	blue	610
50 »	blue	610
64 »	blue	610
4 hours	deep blue	610

New York City) which was not soluble in cold water and which showed retrogradation from solution.

When an aliquot of the reaction mixture (3) was tested with iodine, the suspended white precipitate stained a blue-violet color.

Solution of the insoluble polysaccharide was effected with hot 0.10 *N* sodium hydroxide. When tested for iodine absorption spectrum, maxima were obtained at 560 m μ , 600 m μ , and 610 m μ , depending on when during the course of the reaction the polysaccharide was isolated.

The iodine-polysaccharide complex of all other available cultures of *Oscillatoria* showed a single peak absorption maximum at 550 m μ (3).

It was decided to follow the reaction over the course of 64 minutes at 32° C, as had been done with all other cultures of this alga. The results of iodine-polysaccharide complex absorption spectra, at all time-intervals from the start of the reaction, are shown in the preceding table.

The results showed that there was a shift in the absorption pattern of the iodine-polysaccharide complex during the initial part of the reaction (cf. Table, »10 minutes to 30 minutes«).

Discussion

Normally, extracts of *Oscillatoria princeps* cause the synthesis of a water-soluble polysaccharide from hexose monophosphate. This polysaccharide has a violet iodine color which shows maximum absorption at 550 m μ (3). The polysaccharide, synthesized by the culture of *Oscillatoria* described, showed a maximum absorption which became steady at 610 m μ . However, during the course of its synthesis, the polysaccharide-iodine spectra shifted from 560 m μ to 610 m μ . This was unlike the usual synthesis of polysaccharide in *Oscillatoria* where there was no shift in the polysaccharide-iodine complex spectra (3).

Since the synthesis of polysaccharide in plants has been shown to involve

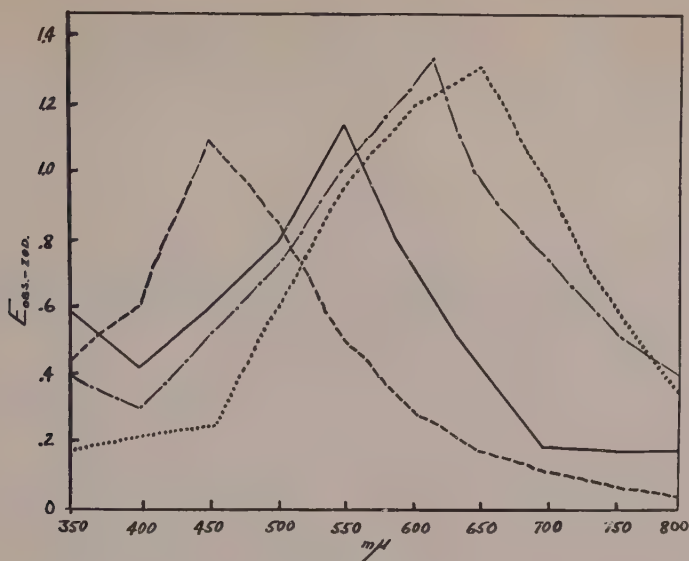


Figure 1. Comparison of Polysaccharide-Iodine Spectra of Various Polysaccharides with Variant of *Oscillatoria*. (---) glycogen, max. absorp. at 420 mμ; (···) amylose, max. absorp. at 650–660 mμ; (----) normal polysaccharide of *Oscillatoria*, max. absorp. at 550 mμ; (-.-.-) polysaccharide variant of *Oscillatoria*, max. absorp. at 610 mμ, similar to amylose.

two types of enzymes (1, 6), one of which forms the *unit chain* by linking glucose residues in α 1 : 4 bonds, and the other of which forms the *branched chain* (1 : 6 bonds), it seemed probable that a variation had occurred in one of these enzymes.

On the basis of recent work (3, 7), it would seem that the polysaccharide produced by this culture of *Oscillatoria* had less *branched chains* (1 : 6 bonds) than usual, and hence, resembled plant amylose (7) in being an almost exclusive *linear* polymer of glucose. As can be seen in Figure 1, the absorption peak of this polysaccharide showed a closer relationship to amylose than to the normal *Oscillatoria* sugar. This in turn, indicated that the enzyme responsible for branching had diminished in activity in this particular strain.

It is suggested that the variation in the type of polysaccharide produced by this culture of *Oscillatoria* was due to a difference in enzyme concentrations or enzyme activity, and that such a difference was brought about as the result of some genic mechanism, possibly a mutation. Such changes have been reported by Brink (2) in higher plants.

Evolution of the algae, in terms of the type of polysaccharide synthesized, may be said to be due to the establishment of such genic changes which

affect the carbohydrate cycle (4). If such variations prove to be of genic origin, they would be *prima facie* evidence of an organized chromatin network in the blue-green algae.

Conclusions

A culture of *Oscillatoria princeps* spontaneously developed the ability to synthesize a type of polysaccharide from hexose monophosphate different from that ordinarily synthesized.

The polysaccharide synthesized by an extract prepared from this culture was insoluble in cold water, and had a blue-violet iodine color which showed maximum absorption at 610 m μ as compared with 550 m μ from previously tested cultures of this alga.

It is suggested that the variation in polysaccharide type may be due to a mutation and, as such, this may be a possible mechanism whereby evolution is effected in the algae.

References

1. Bernfeld, P. & Meutemedian, A.: Isophosphorylase. — *Nature* 162: 297. 1948.
2. Brink, R.: Studies on the physiology of a gene. — *Quart. Rev. Biol.* 4: 520. 1929.
3. Fredrick, J.: Preliminary studies on the synthesis of polysaccharides in the algae. — *Physiol. Plant.* 4: 621. 1951.
4. — Evolution of polysaccharide synthesis in the algae. — *Biol. Rev. of City College* 14: (to be published 1952).
5. Gerloff, G., Fitzgerald, G. & Skoog, F.: Isolation purification, and culture of blue-green algae. — *Am. J. Bot.* 37: 216. 1950.
6. Haworth, W., Peat, S. & Bourne, E.: Synthesis of amylopectin. — *Nature* 154: 236. 1944.
7. Swanson, M.: Iodine color of polysaccharides. — *J. Biol. Chem.* 172: 825. 1948.

Some Effects of Radio Frequency Irradiations on Small Oilbearing Seeds

By

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Plants and seeds have been irradiated infrequently with radio frequency waves (hereafter called »R. F.«), even though they are well suited for many biological observations. Seeds were used in this investigation because they represent autonomous metabolic entities which can be manipulated easily in large numbers. Their degree of germination served as an index of growth response to R. F. treatments. Subsequent carbohydrate analyses indicated certain metabolic effects.

A short review of the literature may serve to gather some widely scattered information. Lower and higher plants have been irradiated since the 1920's (1, 6). Siniuk could increase the germination rate of carrot and onion seeds with 52 Mc/s waves from 32 to 63 per cent and from 30 to 82 per cent respectively. He, nor any other worker, indicated any R. F. power, energy, or potential values. Kuperman claimed earlier blooming of irradiated cucumber seeds at 39 Mc/s, and earlier maturity of irradiated tomatoes and cotton. Pospelow, working with 43 and 60 Mc/s, found an increased germination rate of milo, oats, and wheat. Mezzadrolì and Vareton (16, 17) treated successfully barley, bean, pea and corn seeds with 107 Mc/s. The percentage of germination was increased considerably in each case. Miva and Saito (19) compared with good results the effects of X-ray, ultra-violet light, infra-red light with those of R. F. waves of 20, 50, and 75 Mc/s on Mung beans. Goubareff (9) stated that a high frequency, a high D.C. potential gradient, and a short exposure time provide a better chance for an improvement of the rate of germination than any other combination of these three factors. Working with frequencies from 14 to 60 Mc/s and plate potentials from 600 to 4,550 V during 5 to 15 secs. he found germination

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results which permit the conclusion that the R. F. exposure time $t = F(1/W, f, V)$ = constant, where W = generator power output, f = frequency, and V = D.C. voltage gradient across the seed mass. Accurate seed temperatures were determined first with a removable needle thermocouple by Gilles (8) while working with barley and oats at 225 Mc/s. An optimal irradiation of $1/4$ to $1/2$ minute by R. F. elevated the seed temperature to less than 30° C. The first high powered irradiation was reported in 1944 by Bevilotti (3) who treated pea, chickpea, lentil, wheat, and vetch seeds at 1 Mc/s at 50 KV and 300 MA for periods up to 30 minutes. Biochemical investigations were rather rare. Ireland reported (by abstract) a breakdown of starch particel as seen under the electron microscope. There are no direct indications that R. F. treatments cause significant changes in the auxin content of seeds.

Methods and Procedures

Oilbearing seeds were selected for R. F. irradiation so that effects on sugars could be investigated without interference from polysaccharides. The principles, construction, and action of the irradiation equipment, as described elsewhere (14), required Petri-dishes, which were filled flush with the compacted seed mass. They were placed between the electrodes with air gaps of 5 to 15 mm above the seed to prevent arking.

A simple infra-red heating arrangement was constructed for comparing the effects of external and internal heat supplies to the treated material. One clear 250 W infra-red globe of conical shape was mounted 1.0 cm away from each side of a 1.0 cm high Petri-dish, so that two lights would shine against eachother through the seed.

Some of the seeds were planted in furrows 5 to 7 mm deep in washed medium river sand or in screened potting soil. The planted seeds were covered with fresh pine saw-dust to form a moisture holding seed cover. The seed beds were prepared in redwood flats and kept out-of-doors or in the greenhouse (fig. 1). The soil beds



Figure 1. *Onion seedlings after an exploratory R. F. irradiation of their seeds at 4 Mc/s for (from right to left) 0.0, 1.2, 3.6, 11.0, and 11.0 secs. 6 days after treatment and planting and 1 day after first emergence. Seed coats visible only in first 3 rows.*

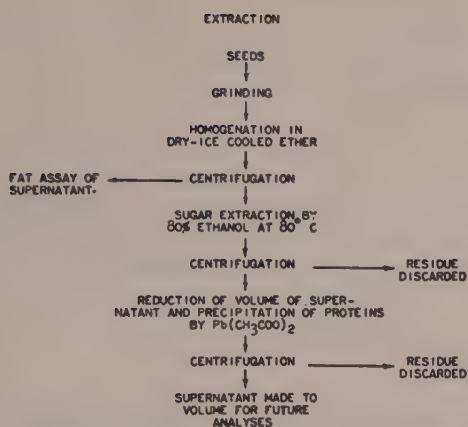


Figure 2. Flowsheet diagram of seed extraction for sugars.

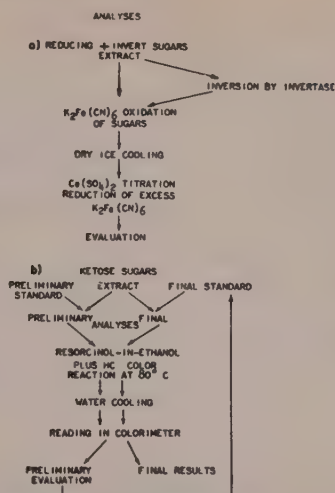


Figure 3. Flowsheet diagram of seed sugar analyses.

were sprinkled daily 1 to 3 times with tapwater; but the sand flats received distilled water until the beginning of seed emergence and then $\frac{1}{4}$ -strength Hoagland's solution (13) and distilled water alternatingly every second day. The number of emerged seedlings was determined daily until it became constant.

Other seeds were analyzed for their contents of fat, glucose, fructose, and reducing sugars after invertase action. The ferricyanide-ceric sulfate method for the determination of reducing sugars (10, 11, 12) was selected because of its inherent simplicity and its practically equal sensitivity for glucose and fructose. The only usable chemical system for the determination of ketoses was the colorimetric resorcinol method (4, 22). Preliminary tests made a modification of these procedures necessary which resulted in a saving of time as shown in the diagrams of figures 2 and 3.

Results

Preliminary experiments suggested an increase of the percentage of emerging carrot and celery seedlings after a few seconds irradiation of dry seeds. Using a D. C. plate potential of 5 KV, an electrode spacing of 20 mm, and two Petri-dishes 14×50 mm and 14×88 mm in size the highest germination rate of Scarlet Nantes carrots, Golden Globe onions, Detroit Perfect beets, and Marglobe tomatoes followed the approximation $V : V' \simeq t : t' \simeq 3.1$ at a fixed electrode distance. V and V' are the volumes of the large and small containers respectively, and t and t' are their best exposure periods. Accordingly, beets should have their highest rate of germination after a 3.4 secs.

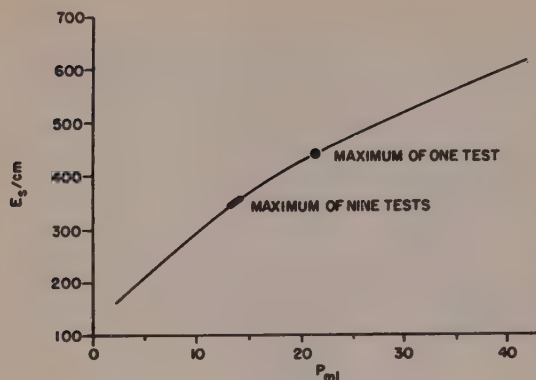


Figure 4. Variation of R. F. power input P_{ml} with R. F. r.m.s. potential gradient E_s/cm across the sample and dependence of germination rate on P_m .

treatment in the small dish between electrodes 20 mm apart. Actually, they showed a germination peak at from 1.2 to 3.6 secs. The optimum time values for other electrode distances depend on the differences between their squared voltage gradients across the sample itself.

While these preliminary experiments were performed on the basis of constant power input at varying lengths of exposure, the following experiments were based on constant periods of exposure with varying power inputs in order to obtain quantitative data about the temperature, power, and energy requirements for maximum seed germination percentages. All seeds of these experiments were planted out-of-doors in potting soil. An abbreviated protocol of an experiment is given in table 1, and figures 4 and 5 summarize the conditions at the highest rate of germination.

A rate of energy input for both carrots and onions of close to 14 W/ml, equivalent to and an energy input of 37.5 cal/ml, corresponded to an exposure time of 11 secs. A potential gradient across the sample of 340 to 360 V/cm

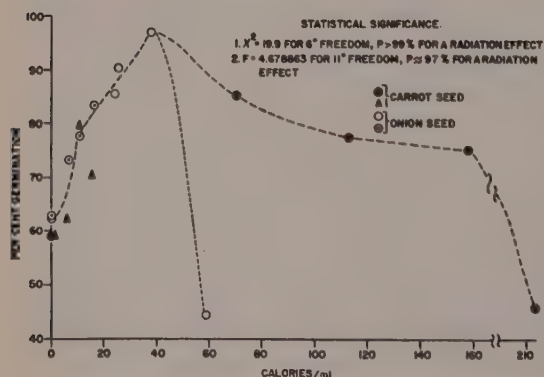


Figure 5. Rate of seed germination as a function of R. F. energy input in calories/ml.

Table 1. *Protocol of a representative R. F. irradiation experiment; Effect of power input variation on seed germination.*

Date: April 14, 1949.

Seed: Southport Red Globe onions with a power factor $\cos \theta = 0.090$, a dielectric constant $K = 3.2$, a density $s = 0.425$, and a specific heat $S = 0.39$.

Irradiation container: Petri dish cover 10×67 mm = 35.25 ml.

R. F. frequency: 45 Mc/s.

Exposure: 3.1 second.

Electrode distance: 15 mm.

Planting: 2 ml = 220 seeds out-of-doors in soil (redwood frames).

Counting: Seedlings counted when coleoptiles were freely visible.

Observation	Day after first emergence	Variable D. C. plate voltage			
		None	3,400	4,700	6,000
T° C.		22.9	30.0	35.3	44.1
W/ml.		0.0	8.7	14.8	21.7
cal/ml.		0.0	6.4	11.0	16.1
Germination %	1	0.9	6.5	2.8	3.7
Zero day =	2	9.3	23.1	23.1	15.7
4 — 25 — 1949	3	27.8	50.9	46.3	43.5
	4	40.7	60.1	50.0	48.1
	5	58.3	73.1	70.3	73.1
	6	62.0	73.1	74.0	81.4
	7	62.9	73.1	77.7	83.3
mg dry weight ¹	21	178	251	208	265
mg dry wt./plant	21	2.7	3.6	3.3	3.3
g fresh weight ¹	119	164	162	149	165
g fresh weight of their bulbs	119	85	94	79	98

¹ All emerged plants minus 5 were dried at day 21. The remaining 5 plants were harvested on day 119.

R. F. — r.m.s. was required for this. Thus, the germination rate can be considered either as a function of power input or time. The time factor can be expressed in two forms by 1) the energy input in calories/ml of seed or 2) the temperature which the seed has reached during the treatment (fig. 6). The highest onion germination of 97 per cent occurred at 50° C while 83 per cent for carrots was obtained at 42° C.

A comparison with carrot seeds treated with infra-red light is very interesting. These seeds had a germination maximum of 71 per cent at 29° C and only 9.5 cal/ml. Any higher energy input reduced their germination drastically. This may mean that heating the whole seed uniformly with a heat gradient towards the interior at temperatures above 30° C is lethal. But temperatures over 50° C from R. F. heating with an opposite temperature gradient are necessary to obtain the same effect.

The treatment caused a reduction of sugars after invertase action and a proportionate increase of ketose sugars at an energy input of already

12.5 cal/ml (fig. 7). An energy input of 20.9 cal/ml did not alter the sugars any further but reduced the fat percentage from 20.0 per cent to 2.6 per cent. The energy input of 12.5 cal/ml corresponded to the steepest slope of figure 5, where the rate of germination is influenced mostly by an input of R. F. energy. The *in vivo* condition of these sugars did not seem to be a requirement for these R. F. effects because irradiated pure sucrose and fructose behaved quite similarly. Here the reducing sugar assay of irradiated glucose increased 10.7 per cent, of fructose 56.8 per cent, and of sucrose 13.8 per cent. The ketose sugar analyses indicated a 54.7 per cent increase for fructose and a 28.5 per cent increase for sucrose.

Discussion

This investigation was an attempt to clarify some of the effects of R. F. irradiations on seed physiology. The recent development of new R. F. techniques and facilities made possible more accurate exposure timing and dosing at high R. F. powers.

The cavity resonator delivered a substantially pure sine wave, and its losses were so low as to be negligible in comparison with the power absorbed by the treated seeds. The peak voltages as delivered by the oscillator were approximately 4 percent below the D.C. potential supplied to the tube. Accordingly the r.m.s. potentials of the sine wave output could be computed easily. The power absorbed by the seeds was found by integrating the product of the current and voltage over that portion of the cycle in which the tube was carrying current. The compacted sample was placed into a central container between the two electrodes of very much larger diameters. The container should not be sealed tightly to prevent moisture condensation in the upper layers and along the container surfaces which would vary the dielectric constants of different portions of the sample mass.

The power absorbed by the materials under treatment, the total energy absorbed, the time of treatment, the voltage gradient, and the final temperature are interdependent. A larger sample or a thicker layer of material may require a greater separation of the electrodes resulting in both a lower overall voltage gradient and a lower gradient through the seeds themselves. This, in turn, results in a reduced rate of power absorption and requires a longer treatment in order to liberate a given amount of energy per ml of seed according to the relation $V : V' \cong t : t' \cong 3.1$.

A determination of χ^2 for the relation of seed germination to energy input (23) according to the $R \times C$ method for quantitative attributes indicated a value of $\chi^2 = 19.9$ for 6° of freedom. This corresponds to a probability of a radiation effect of better than 99 %. A second statistical analysis was made by assuming that the expected number of germinated seeds is a smooth

function of the number of calories/ml according to $E(y) = a_0 + a_1x + a_2x^2 + a_3x^3$. A test of the hypothesis that the number of seeds germinated is always equal to the control value, no matter what the number of calories may be, produced an F-value of 4.678863. This corresponds to a probability for this hypothesis of approximately 3 per cent for $(14-3)^{\circ}$ of freedom in spite of one exceptionally low value in figure 5.

An energy of 37.5 cal/ml was at maximum effectiveness when it was delivered at the rate of 14 W/ml across an average r.m.s. potential gradient of 350 V/cm of seed mass during 11 secs. This time of exposure was of the same order of magnitude as that used by some Russian workers (1) and by Goubareff (9), but somewhat shorter than Gille's exposure (8) of 15 to 30 secs. The fact that ketosugars increased at the expense of inverted sugars at 12.5 cal/ml may indicate that $25.0 = 37.5 - 12.5$ cal/ml were utilized in other processes.

Whereas Goubareff obtained 100 per cent germination with 5 to 15 secs. exposures of unspecified carrot and tomato seeds on moist filterpaper, these experiments produced an increase of carrot germination of 41 per cent, from 59 to 83 per cent. Onion germination increased 54 per cent, from 63 to 97 per cent, and those of tomatoes and beets similarly. These results cannot be compared with those of the Russian investigators because of their use of apparently inferior seeds. The germination curves indicate also that the greatest effect of irradiation was a delayed one. It appeared most strongly at and after the median day of emergence. A delay of 12 weeks between treatment and planting of the various seeds did not reduce the effectiveness of the R. F. irradiations.

The frequency used in this investigation, 43 to 44 Mc/s, lies within the band of 10 to 300 Mc/s which was used by other investigators (1, 2, 7, 9, 16, 19). The distribution of energy of this range within the various seed tissue layers depends on the dielectric constant of these layers and their components, their leakage resistance, and the resonance and polarization properties of their component macromolecules. The endosperm and embryo have a higher constant, and their macromolecules are freer to polarize in an R. F. field than those in the cellulose seed coat. Hence, they act to concentrate or focus the lines of force of the electrical field and thus tend to localize the absorption of energy in the interior of the seed, and there again in certain loci where chemical alterations can be expected to have the greatest effect on metabolic processes.

Previous chemical analyses of irradiated plant material for carbohydrates indicated a decrease of starch and an increase of »sugar» in wheat seeds (1) and a physical break-up of starch granules (Ireland). The heating and resonance phenomena during R. F. irradiations point to such effects, but it

remains to be investigated along which paths these reactions do proceed. Heating of hydrated molecules would lead in all probability to some kind of dehydration and possibly to some type of polymerization. Such temperature dependent reactions would be paralleled by polarization and resonance phenomena. Polarizations may tend towards a rearrangement of carbohydrate molecules with respect to each other which may lead eventually to the formation of molecular aggregates oriented along the axis of the impressed electrical field. This orientation, in turn, will be modified by the configuration and magnitude of the molecular dipoles. A proper value of the dipole moment in relation to the physical dimensions of a molecule will cause its resonance with the radio frequency field. This resonance can be sufficiently strong to break up polarization and heating aggregates. The equilibrium (individual molecules \rightleftharpoons molecular aggregate) can be shifted by varying the relations between these above factors. Also, polymers or condensations, which had existed prior to the R.F. treatment, may be rearranged or changed in size as well. The overall result of the interplay of these factors will be a mixture of carbohydrates of various stages of dehydration, condensation, and realignment.

The present chemical analysis bears out this deduction, for the approximate doubling of the total reducing sugars in carrot and onion seeds from an average of 2.27 to 4.46 per cent was accompanied by an approximate halving of ketose sugars in carrots from 2.78 to 1.20 per cent and in onions from 4.93 to 2.14 per cent. Yet their uninverted reducing sugars did not change during the R. F. treatment. Hence, in accord with Pringsheim (20, 21), Micheel (18) and Dore (5), a condensation and dehydration of invertable reducing sugars could have taken place. The formation of anhydride-oligosaccharides may be visualized by the generation of a lactone ring between C_1 and C_x of one of the invertable hexose units. An internal acetal would result. An alternative to this mechanism would be the formation of an oxygen bridge from C_1 of such a hexose unit to C_x of a second one, thus giving rise to a hexose polymer. In either case, the potentially reducing aldehydic group did not show any unmasked reducing activity after invertase action on the seed extract. Evidently, the R. F. irradiation destroyed β -h-fructoside linkages.

The many reactions possible in such a diverse dehydration process occurring during a very short time interval at unknown molecular temperatures may need about 3.500 Cal/mole for the loss of one H_2O from a hexose. 6.200 Cal/mole would be required for the formation of non-reducing disaccharides from hexose units. Energies introduced into the seeds amounted to 23.80 and 26.75 cal/g, which corresponded to 8.160 and 9.180 Cal/mole of sucrose and to 4.280 and 4.820 Cal/mole of glucose.

Summary

Seeds of carrots, onions, beets, lettuce, and tomatoes have been irradiated with high powered radio waves of 43 to 44 Mc/s. This caused an increased rate of germination which was dependent on the voltage gradient, power and energy input, and temperature of the seed. Infra-red irradiation produced smaller increases of the germination rate. This was paralleled by an approximate halving of total reducing sugars after invertase action on seed extracts. Ketose sugars, in contrast, doubled simultaneously during an energy input of at least 12.5 calories per milliliter of seed.

This paper is part of a dissertation (15) submitted to the Graduate Division of the University of California in partial satisfaction of the requirements for the degree of Doctor of Philosophy.

This investigation was performed with the generous help of Dean Alva R. Davis, Professor of Plant Physiology. The valuable advice in physiological and technical matters of Professors John E. Gullberg and Perry R. Stout are appreciated with gratitude.

Error: In figure 3 line 6 from the bottom should read: PLUS HCL.

References

1. Ark, P. A., and Parry, W.: Application of high frequency electrostatic fields in agriculture. — *Quart. Rev. Biol.* 15: 172. 1940.
2. Bellinzaghi, F.: Azione di un campo a radio frequenza (107 Mc/sec) su la germinazione, I & II. — *Atti Soc. Ital. Sci. Nat.* 80: 226. 1941.
3. Bevilotti, V.: Azione biologica delle corenti ad alta frequenza, I. Influenza esercitata sulla germinazione dei semi. — *Soc. Ital. di Biol. Sperm.* B 19: 258. 1944.
4. Brown, A. H.: The carbohydrate constituents of *Scenedesmus* in relation to the assimilation of carbon by photoreduction. — *Plant Physiol.* 23: 331. 1948.
5. Dore, W. H.: Personal communication. — 1949.
6. Gelders, J. S.: Bibliography of applications of electronics to agriculture. — Committee for Research of Electricity in Agriculture, University of California, College of Agriculture. 1947.
7. Gilles, E.: Un émetteur d'ondes très courtes, de haut rendement, destiné à des recherches biologiques. — *Ann. de l'Univ. Lyons*, 3 B II: 7. 1940.
8. — Sensibilité des graines et plantules aux ondes très courtes. — *Bull. Mens. Soc. Linn. Lyons*. 10: 115. 1941.
9. Goubareff, A. N.: The high frequency oscillator: Its design and use in the study of stimulating and lethal effects on certain biochemical materials. — Master's Thesis, University of California. 1938.
10. Hassid, W. Z.: Determination of reducing sugars and sucrose in plant materials. — *Ind. & Eng. Chem., Anal. Ed.* 8: 138. 1936.
11. — Determination of sugars in plants by oxidation with ferricyanide and ceric sulfate titration. — *Ibid.* 9: 228. 1937.
12. — and Hoagland, D. R.: Syllabus RR, University of California. 1947.

13. Hoagland, D. R. and Arnon, D. I.: The waterculture method for growing plants without soil. — Calif. Agric. Expt. Stat. Circ. 347. 1938.
14. Jonas, H.: Radio frequency irradiation of seeds, principles, procedures, and dosimetry. — (submitted)
15. — Some effects of very high radio frequency irradiations on the germination and metabolism of certain small seeds. — Ph. D. Thesis, University of California. 1950.
16. Mezzadrolì, G., and Vareton, E.: Provo do confronto fra l'azione esercitata delle onde elettromagnetiche ultracorte e del circuito oscillante Lakhowsky, sulla germinazione dei semi e sull' scresimento della piante. — Atti Reale Accad. Nat. di Lincei. 6: 10: 289. 1929.
17. — — Azione esercitata delle onde elettromagnetiche ultracortè sul potere catalasico dei semi. — Atti Reale Accad. Nat. di Lincei. 6: 11: 429. 1930.
18. Micheel, F.: Chemie der Zucker und Polysaccharide. Pages 151, 242. Leipzig. 1939.
19. Miva, H., and Saito, K.: The effect of ultra-short waves on the germination of plants. — Nagoya Journal of Med. Sci. 13: 351. 1940.
20. Pringsheim, H.: Zuckerchemie. — Page 169. Leipzig. 1925.
21. — The chemistry of the monosaccharides and of the polysaccharides. — Page 59. New York. 1932.
22. Roe, J. H.: A colorimetric method for the determination of fructose in blood and urine. — J. Biol. Chem. 107: 15. 1934.
23. Snedecor, G. W.: Statistical methods. — Ames. 1946.

Rapports entre la grandeur des feuilles et le comportement physiologique chez les xérophytes

(Note préliminaire)

par

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Introduction

La dégradation progressive du sol qui a suivi l'expansion humaine a provoqué un changement marqué de la flore et de la végétation du monde entier. Dans la région méditerranéenne française, dans laquelle nous avons procédé à nos expériences écologiques, cette destruction a débuté bien avant l'époque romaine. Autrefois occupées par des forêts denses de Chêne Yeuse, (*Quercus Ilex*) ses collines se trouvent aujourd'hui couvertes d'une végétation pauvre de sous-arbrisseaux épars. Le déboisement et l'altération profonde du microclimat qui en a été la conséquence, ont provoqué un changement radical dans la flore et la végétation primitive. Cette dernière, principalement composée d'espèces ligneuses à feuilles de grandeur moyenne a cédé la place à des groupements de plantes à feuilles de taille très réduite.

Cette substitution des espèces leptophylles¹ à des plantes à feuilles de

¹ On sait que ces termes furent créés par Raunkiaer (22) afin de distinguer des groupes de plantes différents, caractérisés par la grandeur des feuilles. Les limites choisies par lui étaient les suivantes:

leptophylles	0	—	25 mm ²
nanophylles	25 mm ²	—	9 × 25 mm ²
microphylles	9 × 25 mm ²	—	9 ² × 25 mm ²

(deux termes liés par un trait d'union sont des moyens termes).

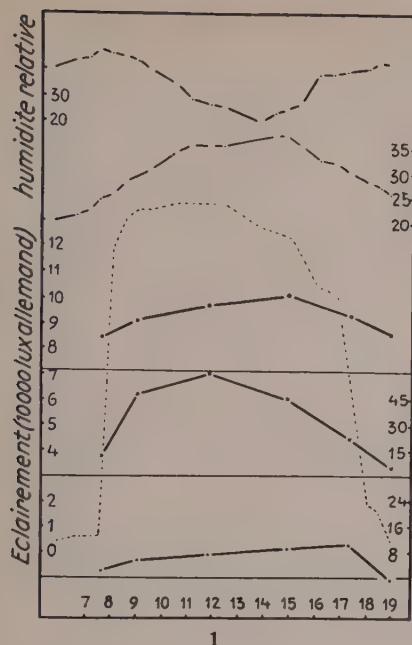


Fig. 1. *Rosmarinus officinalis* L. (après la pluie), leptophylle.

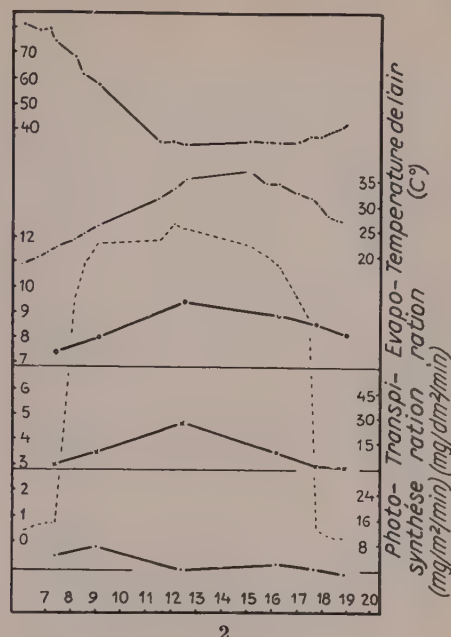


Fig. 2. *Rosmarinus officinalis* L. (pendant la sécheresse), leptophylle.

Légende pour les courbes journalières (heures GMT. sur l'abscisse) de haut en bas sur les graphiques:

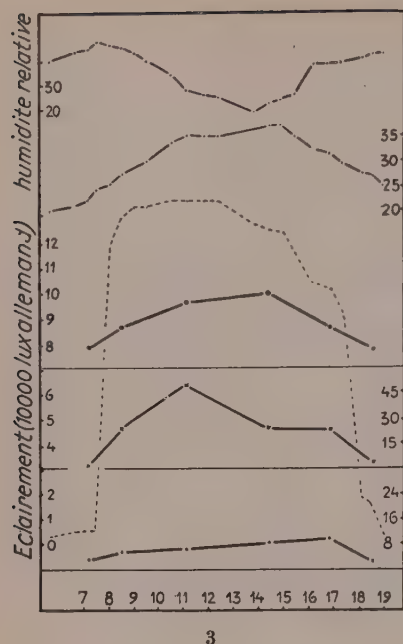
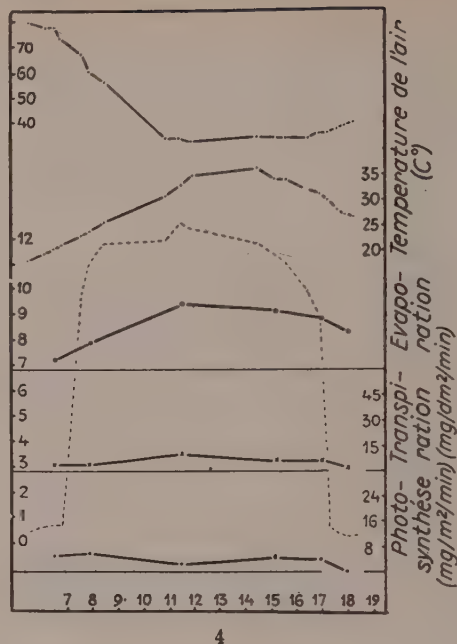
- — ■ humidité relative de l'air.
- — ● température de l'air (en C°)
- éclairage (en 10 000 lux allemand.)
- — ○ évaporation.
- × — × transpiration (en mg/dm²/min.)
- — ● photosynthèse (en mg/m²/min.)

taille moyenne, parallèle à un changement radical des conditions microclimatiques, pose des problèmes d'un intérêt particulier.

Dans le présent travail nous avons essayé d'éclaircir la question des rapports entre la grandeur des feuilles et le comportement physiologique des xérophytes, en partant d'une comparaison des courbes journalières de transpiration et de photosynthèse chez des plantes leptophylles, nanophylles et microphylles de la garrigue narbonnaise.

Choix de la localité et des plantes en vue des mesures

Afin d'effectuer des mesures permettant une comparaison des courbes établies pendant des saisons différentes, il nous a fallu trouver un endroit où le climat était

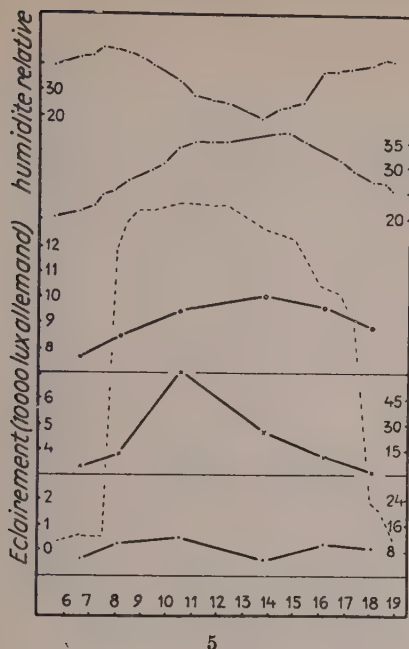
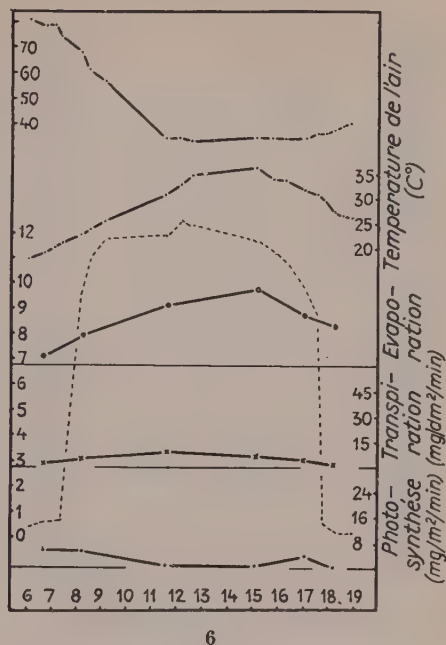
Fig. 3. *Staehelina dubia* L. (après le pluie), leptophylle.Fig. 4. *Staehelina dubia* L. (pendant la sécheresse), leptophylle.

à la fois caractérisé par de grandes similitudes et de grands contrastes. Similitudes quant à la lumière, à l'humidité de l'air et à la température, contrastes quant à l'humidité du sol. Aussi était-il nécessaire d'avoir de nombreuses journées sans nuages pour éviter des effets incontrôlables causés par des changements de l'intensité lumineuse. Il était de première importance également, d'éviter des fluctuations fortes et irrégulières de la tension du gaz carbonique de l'air.

Ces conditions étaient remplies par le choix d'une localité dans un petit massif: la Clape, dans la région narbonnaise. Cette dernière, en effet, se prête d'une façon particulièrement heureuse aux mesures écologiques:

Son climat lumineux, caractérisé par deux saisons nettement distinctes, printemps aux pluies torrentielles durant jusqu'au mois de juin, été d'une sécheresse extrême des mois de juillet et d'août, convient parfaitement aux recherches portant sur le facteur eau. D'autre part ses hautes pressions barométriques, assurant de nombreux jours sans nuages, rendent possibles des comparaisons entre des mesures faites pendant des journées différentes. La stabilité de la tension du gaz carbonique dans cette région, surtout pour les mesures des échanges gazeux, est particulièrement satisfaisante; les fluctuations se sont montrées en effet inférieures à 1 %, ce qui est probablement dû à la proximité immédiate de la mer, laquelle, peut, comme on le suppose, exercer un rôle de tampon; l'absence d'une végétation dense peut également être mise en cause.

Les pentes marneuses du Massif exposées au Sud et jouissant d'une très longue

Fig. 5. *Globularia alypum* L. (après la pluie), nano-leptophylle.Fig. 6. *Globularia alypum* L. (pendant la sécheresse d'été), nano-leptophylle.

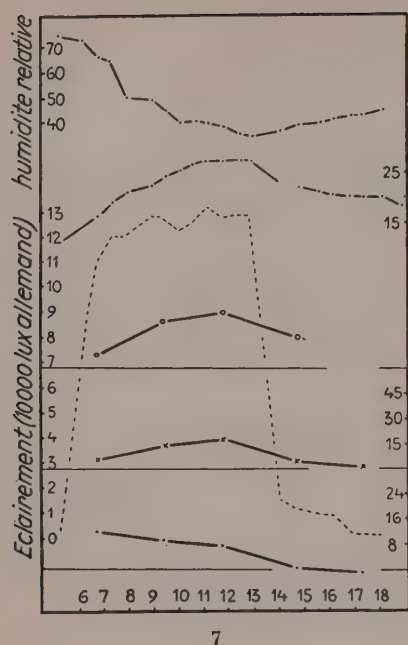
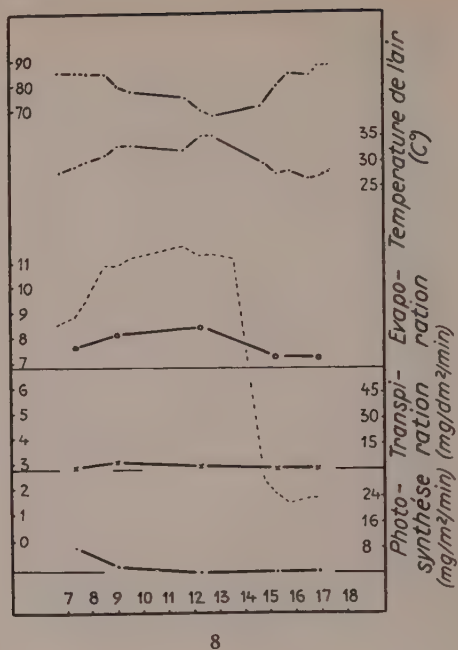
durée d'insolation diurne sont couvertes de landes à Romarin (notamment du Rosmarineto-Lithospermetum staehelinetosum) caractérisées par des sous-arbrisseaux leptophylles. Les pentes exposées au Sud-Est et, en grande partie recouvertes d'éboulis, portent une brousse dense à *Coronilla glauca* et *Bupleurum fruticosum* (Cocciferetum coronilletosum) composée principalement de buissons à feuilles de taille moyenne. Située au pied des falaises verticales, cette localité est à l'ombre assez tôt dans l'après-midi (cf. 2, 5).

Des deux groupements mentionnés, le premier se rapproche le plus, du point de vue écologique, de la forêt primitive; le deuxième par contre, représente un stade de dégradation avancé de la végétation originelle.

Pour étudier le comportement des plantes à feuille de taille très réduite, nous avons choisi les deux espèces leptophylles suivantes qui appartiennent au premier groupement: *Rosmarinus officinalis* L. (rég. médit., nano-phanérophyte), *Stachelia dubia* L. (rég. ouest-médit., nano-phanérophyte).

Pour savoir si le comportement des plantes étudiées est lié à la largeur de leurs feuilles ou à leur station, nous avons choisi l'espèce à feuilles les plus grandes du Rosmarineto-Lithospermetum staehelinetosum: *Globularia alypum* L. (rég. médit., nano-phanérophyte, nano-leptophylle).

Parmi les espèces à feuille de grandeur moyenne du Cocciferetum coronilletosum nous avons utilisé: *Bupleurum fruticosum* L. (rég. médit., nano-phanérophyte, microphylle), *Coronilla glauca* L. (rég. médit., nano-phanérophyte, nanophylle), *Teucrium*

Fig. 7. *Bupleurum fruticosum* L. (après la pluie), microphyllle.Fig. 8. *Bupleurum fruticosum* L. (pendant la sécheresse), microphyllle.

flavum L. (rég. médit., chaméphyte, nanophylle). Cette dernière espèce, en général rupicole, est très fréquente parmi les *Coronilla* et les *Bupleurum*. Elle a été choisie dans le but de savoir si un milieu différent du milieu habituel, a des conséquences sur le comportement physiologique du végétal.

Discussion des techniques

Photosynthèse apparente

Les nombreuses mesures en plein air de photosynthèse effectuées sur des plantes non coupées, dans des régions climatiques différentes notamment en Eurasie et en Afrique du Nord (4, 10, 12, 16, 17, 18, 29) révèlent souvent de fortes fluctuations de courte durée dans l'absorption de CO_2 , fluctuations accompagnées quelquefois même de forts dégagements de gaz carbonique.

L'absence d'indications sur de tels dégagements au cours des expériences faites avec soin au laboratoire, indique (1, 13), semble-t-il, qu'ils sont le fait d'une erreur expérimentale.

Or, dans l'impossibilité de déterminer l'activité photosynthétique d'après les dégagements d' O_2 , nous nous sommes efforcés de réduire l'effet de tout facteur pouvant

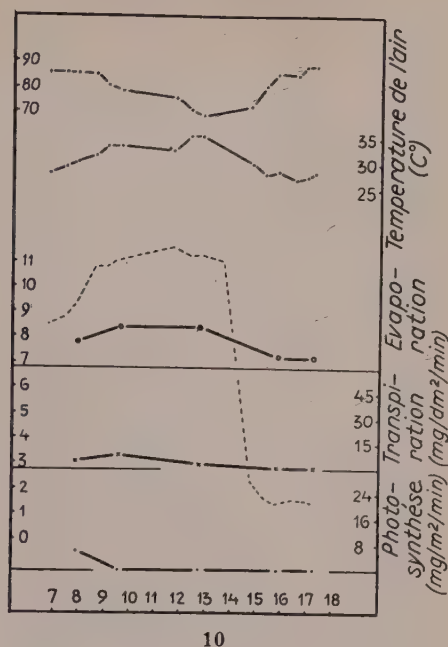
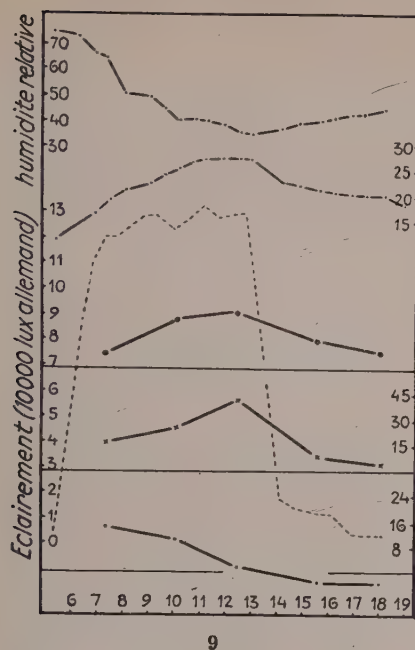


Fig. 9. *Coronilla glauca* L. (après la pluie), nanophylle. (Une coupure accidentelle de la tige à 15 heures exigeait un remplacement rapide avec un autre individu. Celui-ci, étant chargé de gousses non mûres, respirait fortement, ce qui explique les dégagements forts de CO_2 .)

Fig. 10. *Coronilla glauca* L. (pendant la sécheresse), nanophylle.

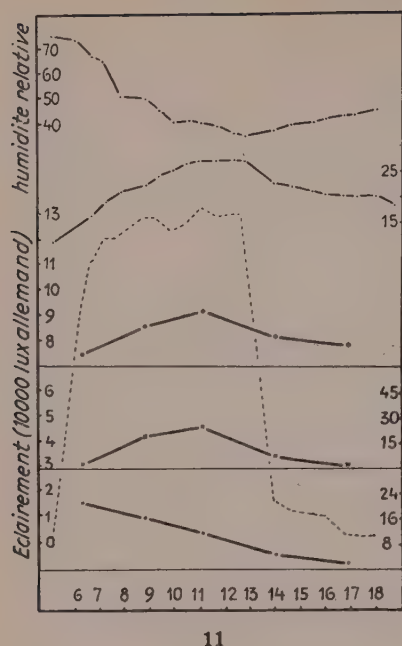
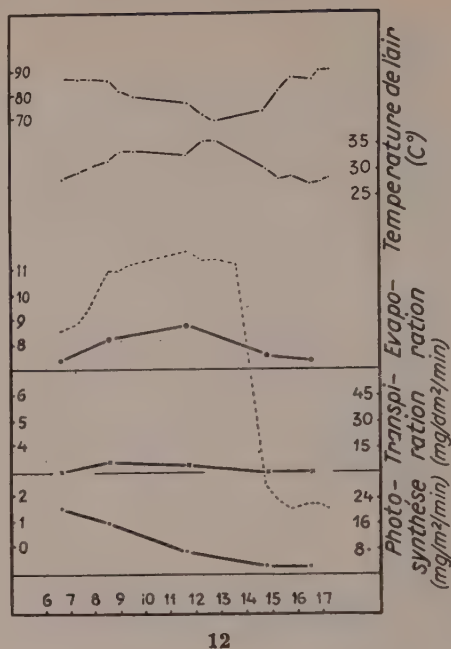
influer sur le coefficient photosynthétique, ou pouvant donner naissance à des dégagements secondaires de CO_2 .

C'est dans ce but que nous avons cherché à éviter tout changement brusque de l'intensité lumineuse avant et pendant les mesures, afin d'éviter tout « effet Emerson » (7); nous nous sommes efforcés également de maintenir la température, à l'intérieur des cloches utilisées pour couvrir les plantes, aussi proche que possible de celle de l'air ambiant. On sait que le phénomène d'absorption de CO_2 à la lumière persiste chez une plante qui a perdu sa faculté d'assimiler à la suite d'un surchauffement et que, ce CO_2 se dégage de nouveau quand la lumière est supprimée (33).

En outre, afin d'éviter des changements dans l'équilibre du CO_2 dissout dans l'eau de la plante, nous nous sommes appliqué à diminuer les fluctuations de la tension de CO_2 sous la cloche avant le commencement des expériences.

Les bouchons fendus, destinés à fermer les cloches, étaient attachés autour des tiges, déjà la veille, pour ne pas provoquer, en touchant la plante, des dommages nuisibles à la photosynthèse (8).

Nous avons jugé comme étant la meilleure, pour nos mesures, la méthode gaz-analytique d'électrotitrage de Thomas (31), adaptée à des mesures en plein air par Stocker, Rehm et Paetzold (29) à laquelle nous avons apporté les modifications suivantes:

Fig. 11. *Teucrium flavum* L. (après la pluie), nanophylle.Fig. 12. *Teucrium flavum* L. (pendant la sécheresse), nanophylle.

Un système de bouteilles de Mariotte assurait un courant d'air continu tant avant, qu'au cours de l'expérience (Des fluctuations inexplicables s'étant produites dans les expériences préalables, où cette précaution n'avait pas été prise). Six litres d'air aspirés à une hauteur de deux mètres entraient en contact avec le végétal non coupé, sous une cloche à double parois (275 cm³) réfrigérées à l'eau froide (écart maximum de la température sous la cloche pendant une expérience: env. 4 degrés). (Des expériences faites pendant l'hiver 1948—49 sans dispositif refroidissant donnèrent naissance à un fort surchauffement à l'intérieur des cloches, suivi par des dégagements considérables de CO₂ en plein jour). Le filtre de verre inséré dans le récipient où s'effectuait l'absorption de CO₂ permettait un passage d'air d'une vitesse de 40 l. à l'heure; (absorption dans une solution de n/100 NaOH à laquelle était ajoutée 2 pour cent d'alcool butylique).

Chaque estimation de l'intensité photosynthétique était déterminée par la différence entre deux mesures consécutives, l'une avec, l'autre sans la plante, et calculée par unité de surface (face supérieure); cette dernière choisie de façon à assurer une absorption du taux en CO₂ de l'air, inférieure à un cinquième (Exposition sous la cloche: 10 min.).

Le pont de Wheatstone, qui était alimenté par un oscillateur électronique, permettait l'estimation d'environ 2000 unités entre la première et la deuxième lecture (sans assimilation). Des changements incontrôlables du volume d'air, pendant l'expérience, déterminés par l'accroissement inévitable de température, provoquaient une erreur d'environ 1 % sur les lectures.

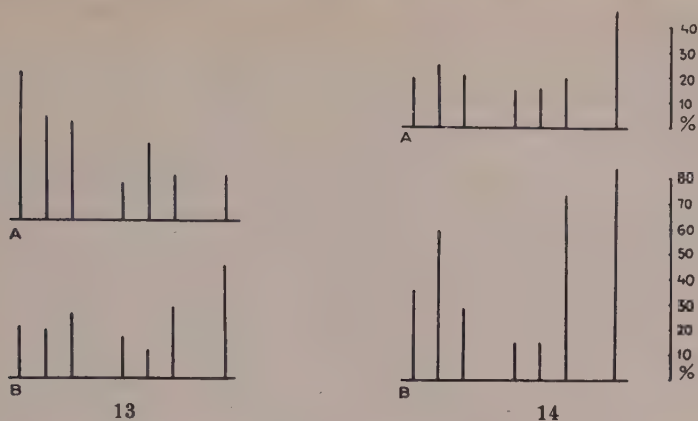


Fig. 13. *Transpiration totale relative d'une journée après le pluie. B. Photosynthèse totale relative d'une journée après la pluie.* (De gauche à droite: Rosmarinus, Stachelina, Globularia, — Bupleurum, Coronilla, Teucrium, — Centaurea.)

Fig. 14. *A. Transpiration totale d'une journée pendant la secheresse en pour cent de la transpiration totale d'une journée après le pluie. B. Photosynthèse totale d'une journée pendant la secheresse, en pourcent de la photosynthèse totale d'une journée après la pluie* (De gauche à droite: Rosmarinus, Stachelina, Globularia, — Bupleurum, Coronilla, Teucrium, — Centaurea.)

Une augmentation de l'humidité de l'air sous les cloches n'a pu être évitée.

Les mêmes individus étaient utilisés pour les mesures après la pluie et pendant la sécheresse à l'exception de Coronilla (cf. légende p. 57).

Tous les résultats ont été déterminés d'après la quantité de CO_2 absorbée par litre d'air sec à 20° .

Transpiration.

La détermination de la transpiration dans des conditions naturelles est fort délicate. Nous n'avons, en effet, que le choix entre deux méthodes, qui toutes les deux ne donnent que des résultats approximatifs. On peut déterminer, ou bien la quantité d'eau évaporée sous cloche d'une feuille non détachée, ou bien calculer la perte de poids d'une feuille coupée.

Etant donnée l'influence prédominante des facteurs externes sur la transpiration et étant donné également le but de nos mesures qui est d'apprécier avec une certaine probabilité la transpiration dans le milieu naturel plutôt qu'indiquer exactement les pertes d'eau dans des conditions anormales, nous avons estimé devoir exclure la première. On sait que l'évaporation dépend du gradient de pression de la vapeur entre la feuille et l'air ambiant, ainsi que de l'épaisseur des couches laminaires d'air humide qui les couvrent (cf. 19) et donc des facteurs: température, humidité et vent, qui se trouvent profondément altérés sous les cloches.

Seule reste donc à notre disposition la méthode de la balance de Stocker (27). Celle-ci est encore la plus employée par les écologistes européens malgré ses défauts;

en particulier le fait de couper la feuille provoque une grave erreur: nous ne savons pas en réalité quel est l'effet des changements de pression de sève qu'elle engendre; nous savons seulement qu'ils peuvent donner naissance à des changements de l'intensité de la transpiration (11, 14).

D'autre part l'exposition nécessairement prolongée des feuilles, entre les deux mesures permet une occlusion non contrôlable des stomates. Ceux-ci réagissent par surcroît d'une façon particulièrement rapide chez de nombreux xérophytes (21).

On se trouve donc en présence de deux sources d'erreurs l'une intervenue au moment de la coupure, l'autre au cours des mesures.

Nous avons essayé d'éviter cette difficulté *en coupant les branches et les pétioles, l'endroit prévu pour la section étant courbé et immergé dans l'huile de Coco, et en réduisant en même temps l'exposition à 2 minutes* (Quelques auteurs russes (15) ont voulu améliorer cette méthode en remplaçant l'huile de Coco par de la paraffine chaude. Or en se solidifiant, la paraffine provoque une obturation absolue des vaisseaux, ce qui à son tour donne naissance à un décroissement rapide de la tension de la sève et ainsi également de la pression des vapeurs des biocolloïdes de la surface de la feuille. Ainsi que l'on pouvait prévoir, les résultats ainsi obtenus sont bien inférieurs à ceux obtenus avec d'autres méthodes).

Toutes les feuilles employées étaient choisies d'avance sur des plantes croissant dans des conditions écologiques quasi-identiques, exposées depuis le matin au rayonnement solaire sans interruption jusqu'à leur récolte.

Etant donnée l'impossibilité de déterminer avec certitude la surface de feuilles ericoïdes ou semblables, nous n'avons utilisé que des feuilles de même grandeur. (Les mesures n'ont d'ailleurs pas la prétention d'être quantitativement exactes, c'est l'allure des courbes qui nous intéresse). Les pesages ont été effectués avec la balance de Bunge (à amortisseur).

Les résultats (moyenne de 3 mesures) sont exprimés, quant aux pertes d'eau, par unité de surface (face supérieure).

La grande similitude de l'intensité de photosynthèse et de transpiration à la même heure pendant des journées différentes (sans changement des forces de succion du sol), toujours constatée pendant les expériences préalables, nous permet, semble-t-il, de faire des conclusions générales d'après les résultats obtenus. Cette reproduction journalière du comportement physiologique a été encore vérifiée au cours des nombreuses mesures, non achevées à la suite d'une apparition de nuages.

Pour les autres mesures nous avons utilisé les méthodes suivantes:

Humidité du sol. — Procédé de Gradmann (9).

Un disque de papier buvard additionné d'une quantité fixe de solution de NaCl et séché à l'étuve en présence de CaCl_2 est exposé au sol dans un récipient, attaché à un crochet fixé sur son bouchon, et l'ensemble tenu à une température constante pendant environ 15 jours. (Afin de diminuer l'espace entre le sol et le bouchon, des disques plats habituellement utilisés pour l'évaporimètre de Piche étaient employés). Les résultats, lus sur une courbe étalon d'après l'augmentation du poids sont indiqués en atmosphères. Pour les sols très humides: méthode cryoscopique. (Les résultats exprimés en atm. à 18°C).

Valeur osmotique. — Méthode de Walter (36, 37).

Les feuilles placées dans des récipients bien bouchés sont chauffées à 100°C au bain-marie pendant $\frac{1}{2}$ heure. La valeur osmotique du jus extrait pas pression est déterminée cryoscopiquement.

Dans certains cas de valeur osmotique élevée (*Globularia alypum*) il a été impossible d'extraire un liquide dépourvu de suspension de tissus macérés, ce qui a empêché une détermination exacte.

Température de l'air. — Thermomètre à mercure installé à 25 cm. du sol, récipient abrité du soleil par un écran placé à 25 cm. de distance deux minutes avant la lecture.

Température des feuilles. — Méthode thermoelectrique d'Eggert (6).

Comparaison entre la température de la feuille et celle de l'air ambiant (l'une des soudures placée dans le parenchyme de la feuille, l'autre exposée à l'air, abritée contre l'échauffement du soleil par un écran placé à une distance de 25 cm.). L'erreur provoquée par l'influence du vent sur ces mesures de courte durée est éliminée si l'on prend la moyenne de 30 mesures (M_{30}). Des fluctuations inévitables de température à l'intérieur du galvanomètre donnent lieu à une erreur de l'ordre de 20 %.

Eclairement. — Appareil photo-électrique de B. Lange.

La cellule toujours exposée perpendiculairement aux rayons solaires. (Tenant compte des changements de la composition spectrale de la lumière au cours de la journée, nous avons essayé de comparer le quotient entre la lumière totale et la lumière filtrée par un écran rouge, comme fonction de l'intensité lumineuse. (L'écran utilisé était Schott & Gen OG 3 non transparent aux ondes inférieures à 546 m μ)).

Humidité de l'air. — Hygromètre à cheveux de W. Koch, Zürich.

L'appareil placé à 25 cm. du sol, abrité du soleil par un écran placé à 25 cm. de distance afin d'assurer la même température à l'intérieur de l'appareil et dans l'air ambiant.

Evaporation. — Evaporimètre de Piche.

L'appareil exposé en plein soleil pendant toute la journée, le disque de papier buvard écarté de 25 cm. du sol. (L'évaporation n'est pas un facteur écologique défini, elle varie suivant le type d'appareil employé. Quelques appareils sont sensibles au vent, d'autres au rayonnement solaire etc. Toutefois il nous paraît vraisemblable que des courbes identiques indiquent des conditions journalières quasi-identiques).

Nous n'avons pas pu trouver une méthode satisfaisante pour déterminer l'ouverture des stomates.

Toutes les mesures sont effectuées *pendant des journées entièrement sans nuages, immédiatement après les fortes pluies*, à la fin de mai et au commencement de juin (les forces de succion des sols, inférieures à 1 atmosphère) et *pendant la sécheresse estivale* au mois de juillet. A ce dernier mois les forces de succion étaient à 40 cm. de profondeur: 165 atm. env. sous *Rosmarinus* et 95 atm. env. sous *Bupleurum*.

Dans les deux groupements végétaux les 3 espèces choisies pour les mesures étaient toutes placées dans un rayon de 80 cm. au maximum (toutes les mesures dans un groupe étaient effectuées pendant la même journée).

Le quotient
$$\frac{\text{lumière filtrée } (> 546 \text{ m}\mu)}{\text{lumière totale}}$$
 (cf. p. 61), était le plus petit à midi et atteignait son maximum à l'aube et au coucher du soleil. Une diminution de moitié de l'intensité lumineuse provoqua une augmentation de 14 % de ce quotient. Une diminution ultérieure d'un cinquième et d'un dixième l'augmenta respectivement de 25 % et de 36 %. Il y avait peu de vent.

Pour la détermination des échanges gazeux nous avons utilisé, ou bien la plante

entière (*Rosmarinus*, *Stachelina*, *Globularia* et *Teucrium*) ou bien le sommet des tiges (*Bupleurum* et *Coronilla*).

L'année 1949 pendant laquelle les mesures ont été faites était caractérisée par une très grande sécheresse estivale.

Resultats experimentaux

1) Valeurs osmotiques (en atm. à 20° C) à 14 heures GMT:

	après les pluies	pendant la sécheresse		après les pluies	pendant la sécheresse
<i>Bupleurum</i>	15,5	40	<i>Rosmarinus</i>	12	38,5
<i>Coronilla</i>	15	42,5	<i>Stachelina</i>	14	50
<i>Teucrium</i>	10,5	37,5	<i>Globularia</i>	19	48

2) Ecart moyen des températures entre la feuille et l'air ambiant à 14 heures GMT au gros de l'été (température de l'air environ 37° C).

<i>Bupleurum</i>	$M_{30} = 7,2 \pm 0,57$	<i>Globularia</i>	$M_{30} = 1,2 \pm 0,23$
<i>Coronilla</i>	$M_{30} = 4,5 \pm 0,65$	<i>Stachelina</i>	$M_{30} = 0,8 \pm 0,18$
<i>Teucrium</i>	$M_{30} = 4,4 \pm 0,52$	<i>Rosmarinus</i>	$M_{30} = 0,3 \pm 0,17$

3) Les mesures journalières de température, d'humidité, d'évaporation, d'éclairement, de transpiration et de photosynthèse se trouvent portées en graphiques (fig. 1—12).

4) Enfin, à la page 59 ont été figurés, d'une part la comparaison entre la transpiration et la photosynthèse totales au cours d'une journée de la saison humide (fig. 13); d'autre part la faculté relative des végétaux à réduire la transpiration et la photosynthèse totales pendant la saison sèche (fig. 14). Ces deux derniers graphiques, intéressent encore une espèce des stations très arides: *Centaures intybacea* Lmk. (nano-leptophylle, rég. est-médit.).

Les faits principaux qui se dégagent de l'examen de nos mesures, peuvent être résumés ainsi:

I. — Pendant la saison sèche il y a une forte réduction de l'intensité moyenne de photosynthèse (fig. 1—10) et de transpiration (fig. 1—12).

II. — Après la pluie les espèces leptophylles (*Rosmarinus* et *Stachelina*) sont caractérisées par l'absence d'une «dépression de l'après-midi» de l'activité photosynthétique (fig. 1, 3).

III. — Une dépression de la transpiration dans l'après-midi n'est pas toujours suivie d'une dépression semblable de l'activité photosynthétique (fig. 1, 3).

IV. — Il existe un rapport direct entre l'écart moyen de température entre l'air et la feuille, et la grandeur des limbes (en été). Cet écart est plus élevé chez les espèces microphylles et nanophylles que chez les leptophylles (pag. 62).

V. — La transpiration totale la plus abondante (par unité de surface) se constate chez les leptophylles et chez les nano-leptophylles. (fig. 13).

VI. — Les espèces qui réduisent le plus fortement la transpiration totale pendant la saison sèche diminuent en même temps fortement leur photosynthèse totale (fig. 14) (sauf *Teucrium*¹).

VII. — Les espèces caractérisées par l'activité photosynthétique totale la plus intense ne se distinguent pas par une transpiration particulièrement abondante (mesures faites après la pluie) (fig. 13).

VIII. — L'écart des valeurs osmotiques entre le printemps et l'été était très prononcé, ce qui met en évidence la très grande différence des conditions hydriques pendant les deux saisons (pag. 62).

Discussion

Les possibilités multiples de dispositifs susceptibles de permettre à une espèce de supporter une saison sèche, ainsi que la grande diversité de structure des xérophytes, résultant en partie de leur origine systématique différente, rendent difficile toute tentative pour les caractériser.

Voilà aussi pourquoi le mot xérophyte a été employé avec des significations et des compréhensions si différentes à travers les temps. Ce terme, au commencement employé dans un sens large, comprenant toutes les espèces végétales croissant dans des stations sèches (24), a été plus tard réservé à des groupes tantôt physiologiques, tantôt morphologiques. Dans le présent travail nous avons employé le terme dans son acception primitive.

Les xérophytes comprennent alors quatre groupes constitutionnels liés au sols secs, à savoir:

I. Les géophytes et les thérophytes, qui survivent pendant la saison sèche, les premiers par leurs parties souterraines, les secondes sous forme de semences.

II. Les plantes succulentes à racines superficielles qui vivent sur leurs réserves d'eau pendant les saisons sèches en diminuant fortement la transpiration ainsi que les échanges gazeux.

III. Les plantes, qui possèdent des racines atteignant l'eau phréatique, et qui continuent une transpiration et une activité photosynthétique (à peu près) normale pendant la saison sèche.

IV. Les plantes n'appartenant pas aux groupes mentionnés ci-dessus, qui cependant survivent aux saisons sèches en assurant un bilan d'eau suffisant, en appliquant une coordination excellente entre les mouvements stomataux et l'absorption des racines.

A en juger d'après les résultats, nos expériences se sont portées notamment sur des espèces s'approchant du dernier groupe.

¹ Au cours des expériences préliminaires *Teucrium* s'était montré doué d'une faculté extraordinaire d'absorption d'eau par les feuilles. Il perd souvent sa turgescence pendant la journée mais se remet très vite après une diminution de la transpiration, comme par exemple pendant l'exposition sous les cloches. Cela explique probablement la faible différence d'intensité photosynthétique constatée entre le printemps et l'été.

Le premier fait qui ressort de l'examen de nos courbes est la forte réduction de la photosynthèse totale en été. Si nous considérons la ressemblance des courbes microclimatiques au printemps et en été, joint au fait que l'écart très prononcé des valeurs osmotiques des deux saisons met en évidence de très grandes différences hydriques, il sera permis de l'attribuer essentiellement aux pertes d'eau causées par l'augmentation considérable des forces de succion du sol pendant la saison sèche. Une telle diminution de la photosynthèse peut être considérée comme l'effet soit d'une occlusion des stomates (25), soit d'une réponse plasmique; cette dernière a surtout été mise en évidence par des expériences sur des plantes dépourvues de stomates (35); une déshydratation semble provoquer une intensification des réactions cataboliques au détriment des réactions anaboliques (28). La diminution même des processus photosynthétiques résulte, peut-être, d'une destruction de la chlorophylle.

Afin d'expliquer la dépression vespérale de l'activité photosynthétique observée chez plusieurs espèces, même pendant la saison des pluies, il nous faut probablement encore envisager l'influence d'une accumulation d'assimilats dans les feuilles. Effectivement, des théories modernes accordent une grande importance à une action régulatrice attribuée à la «cat C», catalyseur supposé nécessaire pour la libération d'O₂, effectuée par un processus de dismutation; une trop forte concentration d'hydrate de carbone semble entraîner une oxydation des matières organiques, créant des acides organiques à effet narcotique, susceptibles d'adhérer à la surface de la chlorophylle et de la rendre inactive (8).

De même que pour la photosynthèse, la diminution de la transpiration totale en été peut être attribuée au manque d'eau; une obturation des stomates semble surtout responsable. Cependant ne faut-il pas sous-estimer l'effet même de la déshydratation des biocolloïdes. Ainsi nombre de mesures indiquent que des facteurs internes, tels que des changements de la pression dans les vaisseaux, peuvent provoquer des changements de l'intensité de transpiration.

La marche journalière de la photosynthèse et de la transpiration chez les xérophytes étudiés semblent ainsi en grande partie réglées par un facteur plasmique. De même on constate chez les deux leptophylles étudiés, que la photosynthèse s'intensifie lorsque la transpiration diminue. Nous savons en effet que l'intensité de la photosynthèse n'est pas toujours la plus forte quand la turgescence est au maximum; une certaine déshydratation semble lui être favorable (3). L'intensification de la photosynthèse pourrait donc s'expliquer par l'effet d'une augmentation du déficit en eau des tissus pendant la journée. (Il existe d'ailleurs de nombreux cas où un rapport entre le degré d'ouverture des stomates et l'activité photosynthétique n'a pu être établi (23, 34)).

Il est intéressant de noter que la transpiration abondante des plantes à petites feuilles (cf. 26), indiquant une communication facile entre l'espace intracellulaire et l'atmosphère (20, 21) n'est pas suivie dans notre cas par une grande intensité de photosynthèse.

Chez les plantes étudiées existe un rapport net entre la largeur des feuilles et l'écart de températures entre l'air et la feuille. Ce n'est cependant pas une règle générale; des mesures effectuées avec d'autres plantes montrent que des facteurs tels que la couleur, l'épaisseur des feuilles peuvent influencer de façon décisive sur la température des feuilles.

La constatation la plus intéressante de notre travail se dégage de la comparaison des courbes journalières après la pluie chez les leptophylles et chez les nanophylles et les microphylles.

La différence de comportement est surtout nette en ce qui concerne l'activité photosynthétique: chez les leptophylles, elle croît peu mais régulièrement pendant la journée, chez les nanophylles et chez les microphylles elle décroît rapidement après un maximum situé très tôt dans la matinée. Globularia alypum (nano-leptophylle) constitue un cas de transition entre ces deux comportements.

Une comparaison de la courbe de photosynthèse de Globularia avec celles de Rosmarinus et Staehelina montre que le comportement particulier des deux leptophylles étudiées n'est pas lié à une station donnée.

Il n'y a pas de rapport non plus entre la répartition géographique des espèces étudiées et leur comportement. La limite septentrionale de Staehelina par exemple passe plus au Nord que celle de Rosmarinus.

Comment donc expliquer ce comportement particulier des plantes à feuilles étroites?

Nous savons déjà depuis longtemps que les effets d'une déshydratation dans une feuille large se marquent d'abord dans ses parties latérales et intravasculaires (30, 38). Nous savons également qu'il existe souvent de fortes différences des forces de succion à travers une feuille (32), ce qui indique la lenteur des migrations d'eau.

Ces faits, joints aux résultats de nos mesures, nous permettent d'entrevoir les qualités essentielles des leptophylles qui leur permettent d'assimiler pendant la journée entière durant la saison humide:

1. *danger de surchauffement nuisible à la photosynthèse diminué.*
2. *migrations des assimilats plus faciles.*
3. *danger de deshydratation des certains tissus, moindre.*

Nos mesures ont mis en évidence que la leptophyllie s'accompagne d'un comportement physiologique spécial. La question qui se pose est de savoir si celui-ci constitue un avantage pour les plantes de sol sec.

Les expériences faites sur des plantes cultivées montrent que l'activité physiologique de certaines espèces est très labile. Ainsi certaines plantes font preuve d'une très grande transpiration dans la matinée, pour l'arrêter au courant de l'après-midi. D'autres au contraire, montrent une grande stabilité et transpirent peu et régulièrement pendant toute la journée, le taux total d'eau perdue étant le même.

De même nous constatons que les leptophylles étudiés, malgré leur activité photosynthétique continue, n'élaborent pas plus de matières organiques par unité de surface que les espèces caractérisées par une forte dépression vespérale. Nous avons observé également qu'elles sont caractérisées par une transpiration très abondante. Non seulement la transpiration par unité de surface est grande, mais les pertes totales d'eau, causées par le très grand nombre de feuilles chez ces espèces, sont également considérables, et nous avons vu que leur pouvoir de réduire les pertes d'eau en été n'est pas plus grand que celui des nanophylles et les microphylles étudiés.

Il nous faudrait donc admettre que la faculté des leptophylles d'assimiler pendant toute la journée durant la saison humide ne proviendrait probablement pas d'une adaptation favorable à un milieu sec, mais serait plutôt une conséquence de la morphologie particulière de leurs feuilles.

Nous n'avons pas constaté des dégagements de CO_2 en plein soleil (cf. lég. fig. 9). Il est possible cependant que de tels dégagement se réalisent dans la nature. Chaque feuille d'une plante est en général exposée à des fluctuations considérables d'éclairement et de température et souvent de tension du gaz carbonique pendant la journée.

Une détermination de la photosynthèse d'après les échanges gazeux, sous une cloche réfrigérée à l'eau, telle que nous l'avons effectuée, permet seulement d'estimer l'assimilation moyenne d'un certain nombre de feuilles placées dans un milieu où les variations naturelles des facteurs ambiants sont fortement réduites.

La diminution brusque de l'intensité lumineuse, vers 14 heures, dans la station du Cocciferetum coronilletosum n'est probablement pas assez grande pour que les questions de photopériodisme entrent en cause. L'éclairement dans l'après-midi n'est pas suffisamment faible pour inhiber la mise en fleur d'une plante de journée longue.

Summary

Broad-leaved and narrow-leaved mediterranean xerophytes, growing under nearly equal micro-climatic conditions, were the object of a comparative study of transpiration rates and photosynthetic activity during the dry and the rainy season.

The measurements, made by improved methods under favourable condi-

tions with cloudless weather, reveal a close relationship between the breadth of the leaves and the physiological behaviour of the plants.

During the humid season, the narrow-leaved plants studied show a day-curve of photosynthetic activity which is nearly linear and slightly ascending whereas the one of the broad-leaved species is distinguished by a decided drop beginning in the early morning. During the dry season photosynthesis nearly stops during the afternoon in all species.

It was further recorded that the highest internal temperatures of the leaves examined were to be found in broad-leaved species.

In general, the species possessing the greatest ability to reduce their total rate of transpiration in summer, also show the most marked reduction of total CO_2 absorption.

Species having the highest total transpiration rates during the rainy season often had the lowest photosynthetic rates during the same period. The highest daily transpiration rate per surface unit was found in narrow-leaved species.

The results were discussed and led to the conclusion that the aptitude of the observed narrow-leaved xerophytes to assimilate during the whole day, during the rainy season, should not be considered a quality of adaptation favourable to life in arid regions and capable of explaining their abundance therein. It is more likely that their special behaviour may be a mere consequence of the structural peculiarities of their leaves.

Bibliographie

1. Boysen-Jensen, P. et Müller, D.: Die maximale Ausbeute und der tägliche Verlauf der Kohlensäureassimilation. — *Jahrb. f. wiss. Bot.* 70: 493. 1929.
2. Braun-Blanquet, J.: La Chênaie d'Yeuse méditerranéenne (Quercion ilicis). Monographie phytosociologique. — *Comm. S.I.G.M.A.* 45. 1936.
3. Brillant, V. A. et Tschrelaschewi, M. N.: Influence of the hydration degree of the assimilatory tissue on the photochemical and dark reactions in photosynthesis. — *Acta Instituti Botanici Academiae Scientiarum U.R.S.S.* Ser. IV: 88. 1940.
4. Drautz, R.: Über die Wirkung äusserer und innerer Faktoren bei der Kohlensäureassimilation. — *Jahrb. f. wiss. Bot.* 8: 171. 1935—1936.
5. Eckardt, F.: Le comportement physiologique de quelques xérophytes de la garrigue narbonnaise. — Thèse, Montpellier. 1951.
6. Eggert, R.: The construction and installation of thermocouples for biological research. — *J. Agric. Res.* 72: 341. 1946.
7. Emerson, R. et Lewis, Ch. M.: Carbon dioxide exchange and the measurement of the quantum yield of photosynthesis. — *Amer. Journ. Bot.* 28: 759. 1941.
8. Franck, J.: Photosynthetic activity of isolated chloroplasts. — *Rev. Mod. Physics* 17: 112. 1945.
9. Gradmann, H.: Über die Messung von Bodensaugwerten. — *Jahrb. f. wiss. Bot.* 80: 91. 1934.

10. Guttentberg, H. von et Buhr, H.: Studien über die Assimilation und Atmung mediterraner Macchiapflanzen während der Regenzeit- und Trockenzeit. — *Planta* 24: 163. 1935.
11. Haines, F. M.: Transpiration and pressure deficit. IV. — *Ann. Bot. N. S.* 1: 283. 1936.
12. Harder, R., Filzer, P. et Lorenz, A.: Über Versuche zur Bestimmung der Kohlensäure-assimilation immergrüner Wüstenpflanzen während der Trockenzeit in Beni-Unif (algerische Sahara). — *Jahrb. wiss. Bot.* 75: 45. 1931.
13. Hoover, W. H., Johnston, E. S. et Brackett, F. S.: Carbon dioxide assimilation in a higher plant. — *Smithson. Misc. Coll.* 87: 1. 1933.
14. Humphries, E. C.: The effects on the rates of transpiration and absorption of small variations in the pressure in the xylem tracts. — *Ann. Bot. N.S.* 2: 665. 1938.
15. Iwanoff, L., Silina, A. et Celniker, J.: Contributions à la méthode de pesage rapide pour la détermination de la transpiration dans des conditions naturelles. (en russe). — *Botanicheskij Journal, Akad. des Sciences U.R.S.S.* 25: 171. 1950.
16. Jaccard, P. et Jaag, O.: Schwankungen der CO₂ Aufnahme bei höheren Pflanzen in CO₂-reicher Luft und kontinuierlicher Beleuchtung. — *Ber. d. Deutsch. Bot. Ges.* 50: 167. 1932.
17. Killian, Ch.: Recherches écologiques *sur l'assimilation chlorophyllienne chez des monocotylédones printanières. — *Rev. gén. Bot.* 45: 1. 1933.
18. Kostytschew, S., Bazyrina, K. et Tschesnokow, W.: Untersuchungen über die Photosynthese der Laubblätter unter natürlichen Verhältnissen. — *Planta* 5: 696. 1928.
19. Leighly, J.: A note on evaporation. — *Ecology* 18: 180. 1937.
20. Maximov, N. A.: The physiological significance of the xeromorphic structure of plants. — *Journ. of Ecology* 19: 273. 1931.
21. — The plant in relation to water. — 2nd. London 1935.
22. Raunkiaer, C.: Om bladstørrelsens anvendelse i den biologiske plantegeografi. — *Botanisk Tidsskrift* 34: 225. 1916.
23. Schneider, G. W. et Childers, N. F.: Influence of soil moisture on photosynthesis, respiration and transpiration of apple leaves. — *Plant Physiol.* 16: 525. 1941.
24. Schouw, J. F.: Grundtræk til en almindelig plantegeografie. — København 1822.
25. Stålfelt, M. G.: Die Spaltöffnungsweite als Assimilationsfaktor. — *Planta* 23: 715. 1935.
26. Stocker, O.: Die Transpiration und Wasserökologie nordwestdeutscher Heide- und Moorpflanzen am Standort. — *Zeitschr. f. Bot.* 15: 1. 1923.
27. — Eine Feldmethode zur Bestimmung der momentanen Transpirations- und Evaporationsgrösse. — *Ber. d. deut. bot. Gesell.* 47: 126. 1929.
28. — Beiträge zu einer Theorie der Dürresistenz. — *Planta* 35: 445. 1948.
29. — Rehm, S. et Paetzold, I.: Beiträge zur Methodik der kurzfristigen Assimilationsmessung. — *Jahrbüch. f. wiss. Bot.* 75: 556. 1938.
30. Thoday, D.: Experimental researches on vegetable assimilation and respiration. V. A critical examination of Sachs method for using increase of dry weight as a measure of carbon dioxide assimilation in leaves. — *Proc. Roy. Soc. B.* 82: 1. 1909.
31. Thomas, Mayer T.: Precise automatic apparatus for continuous determination of carbon dioxide in air. — *Ind. eng. chem. anal. edit.* 5: 193. 1933.
32. Ursprung, A. et Hayoz, C.: Zur Kenntnis der Saugkraft. VI. Weitere Beiträge zur Saugkraft des normalen und abgeschnittenen Hederablattes. — *Ber. d. deut. bot. Gesell.* 40: 368. 1922.
33. Veen van der, R.: Inductive phenomena in photosynthesis. I. — *Physiol. Plant.* 2: 217. 1949.

be adequately characterized by physical evaporation measurements only, we must resort to the more complex method of attending to each factor separately.

(1) *Atmospheric humidity* — The rate of evaporation from the moist surface of a physical body is proportional to the difference between the vapour pressure at the evaporating surface and the vapour pressure of the surrounding atmosphere (cf. Leighley 1937). If this difference be kept constant, temperature variations do not affect the evaporation rate.

The application of this law to transpiration experiments demands caution, since a living plant is a system the properties of which are very different from those of any physical object. To begin with, the water vapour pressure of the atmosphere can be easily calculated from temperature and relative humidity data, but the vapour pressure at the transpiring leaf surface is not so readily accessible to measurements, especially in field experiments. When the necessary measurements of temperature and humidity at the leaf surface are lacking, as is most often the case, a calculation of the vapour pressure difference must be based upon gross assumptions. The most usual procedure is to presume the temperature of the leaf surface to be equal to the atmospheric temperature, and the relative humidity at the surface to be 100 %. The vapour pressure difference between surface and air would then become equal to the saturation deficit of the atmosphere (cf. Anderson 1936). If these assumptions were justified, the transpiration intensity would thus be proportional to the saturation deficit of the atmosphere, and the reduction of different observations would become an exceedingly simple affair.

The extensive investigations of Gäumann and Jaag (1936, 1938) have shown, however, that the influence of humidity variations upon transpiration is actually much more complicated. Even if the atmospheric temperature is kept constant and the temperature deviations of the transpiring leaf are taken into account by calculation of the so-called »physiological saturation deficit», (Gäumann and Jaag 1936, p. 445), no linear relationship can be shown to exist. Moreover, striking individual variations often occur within the same species.

This means that, whichever method we may choose for comparing transpiration values, it is encumbered with inherent errors, and that we must resort to approximate methods which minimize these errors as far as possible.

The investigations of Gäumann and Jaag (l.c.) have shown that the most serious irregularities in the course of transpiration curves occur at high transpiration intensities, i.e., at high temperatures in dry air. Consequently, experiments intended to serve as a basis for comparison between different species should preferably be carried out under conditions which induce only a moderate transpiration. When no measurements of leaf temperatures can be made during the experiments, this precaution also becomes necessary in order to keep the inevitable deviations from atmospheric temperature within reasonable limits.

An examination of the many transpiration curves published by Gäumann and Jaag shows further that under such conditions, e.g., in the range of a physiological saturation deficit of about 5 mm Hg, the curves for both cuticular and stomatal transpiration can, for a short distance, be quite satisfactorily approximated by straight lines. Provided that only observation points which lie within this range be used for comparison, reduction by a simple proportional relation may, therefore, in many cases, give sufficiently accurate results for ecological and physiological purposes, if the formulation of exact relations is not intended.

The Photoperiodic Behaviour of Short-day Plants

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It is well-known that the effects produced on photoperiodically sensitive plants by long days (accelerated flowering of long-day plants, postponement of the flowering of short-day plants) may be equally well obtained by subjecting them to a few hours of light each day, and interrupting the following long dark period by a very short »light break» which may be of relatively low intensity. The effectiveness of such a light break is maximal when it occurs about or a little after the middle of the daily dark period (Hamner and Bonner, 1938; Naylor 1941; Harder and Bode, 1943; Bünning, 1944 b; Parker *et al.* 1946; Claes and Lang, 1947). Sensitivity to a light break increases up to this point during the dark period and declines from this point onwards.

In 1947 Claes and Lang published the results of experiments in which the long-day plant, *Hyoscyamus niger* was subjected to photoperiodic treatments in which each cycle of light and darkness totalled 48 hours. The 41 hours of darkness which followed each 7 hour »main» light period was interrupted at different points by a subsidiary light period (or »light break») lasting 2 hours. The results showed that with regard to flowering there are two optima of the effectiveness of the subsidiary light period, the first lying about 16 hours after the beginning of the main light period, the second optimum about 24 hours after the first. Between these points the subsidiary light period had less effect in promoting flowering. Plants receiving a subsidiary light period 21 to 36 hours after the beginning of each cycle did not flower. Claes and Lang interpreted their data in terms of the theory of the mechanism of photoperiodism proposed by Lang and Melchers (1943) and also in terms of the theory of Bünning (1944 a, 1944 b, 1946).

No similar experiments have been reported for short-day plants, despite the importance of such experiments in comparing various theories of photoperiodism. The results of some experiments with *Perilla ocymoides* and *Xanthium* are reported in this paper, and the behaviour of these short-day plants is compared with predictions which may be made on the basis of various theories of photoperiodism, particularly those of Gregory (1948) and Bünning.

Materials and Procedure

The experiments were carried out during 1950–1951 on the short-day plants *Perilla ocymoides* and *Xanthium pennsylvanicum* (the original material was determined by Blake as *X. saccharatum*, see Parker *et al.* 1946). *Perilla* seed was obtained from a stock grown for several generations at Manchester. *Xanthium* seed was obtained from plants raised by Professor F. G. Gregory from seed supplied by Professor K. C. Hamner. It has been found in previous experiments that plants of the *Perilla* stock do not flower if subjected to more than 12 hours of daylight per day. The photoperiodic reactions of the *Xanthium* plants are presumed to be the same as those of plants used in experiments carried out by Hamner (1940). Prior to experimental treatments all the plants used were raised under long-day conditions in a greenhouse. When the length of natural daylight fell below 16 hours supplementary illumination from a 5 ft. 80 watt daylight-type fluorescent lamp was used to prolong the daylength to 16 hours.

Seeds of *Perilla* were sown in flats and seedlings pricked out and transferred to 3" pots: at or before the time of experimental treatment they were re-potted to 4" pots (except in Experiment 1). *Xanthium* seeds were sown in flats and the seedlings transferred to 4" pots. In experiments carried out in winter and early spring the plants were given light treatments in a growth cabinet designed and constructed by Dr. P. F. Wareing. In this cabinet eight 5-ft 80-watt daylight-type fluorescent lamps provide an intensity of illumination of not less than 1200 ft. candles at the tops of the plants. (Measurements of light intensity were made throughout with a calibrated barrier-type photocell, held at right angles to the direction of incident light. For a comparison of the spectral composition of the light of the fluorescent lamps with daylight see Wangermann and Ashby, 1951.) The temperature in the cabinet is thermostatically controlled and was maintained during the experiments at 18° C. At the end of the light treatments the plants were placed under dark boxes in the room which houses the cabinet. Although the temperature in this room is not controlled, it is partly below ground and temperature fluctuations are reasonably small.

During the summer, daylight was used to provide the main light period, and the plants were then removed to dark cupboards. In these experiments the temperature was not controlled either during the light periods or during the dark periods. The intensity and duration of the light applied during the light breaks is indicated in each case in the detailed descriptions of the experiments.

and cannot easily be determined in advance for each object, this value cannot be employed as a standard condition.

Besides, Stålfelt has shown in other experiments (1932 b) that minor variations of aperture in stages of widely open stomata seem to have only small influence on the transpiration rate. Even if the complete saturation prescribed here, should tend to produce slightly submaximal stomatal apertures, this effect ought therefore not to interfere seriously with the validity of the results obtained.

Finally, it may be inferred from the theory of transpiration decline in excised objects, which we are going to discuss in the following chapter (III), that the formula employed there to describe the quantitative relationship between water content and transpiration rate cannot be expected to hold good in the range of low water contents. For this reason also, the transpiration experiments ought to be started at the stage of complete saturation.

Outline of experimental procedure

The precautions in method indicated above, aim at eliminating as far as possible the influence of accidental factors in comparative transpiration experiments, in order that the possible correlation between individual structure and transpiration rate may not be masked by the complex pattern of variable factors which are involved in the water balance of plants under natural conditions. It ought to be evident from the preceding considerations, that this intention cannot be realized in practice with the exactness of a physical experiment. Even taking into account the standardization of external conditions alone, truly exact experiments would require a constant pressure cabin with automatic temperature and moisture control, artificial illumination, etc., equipment which as yet is not available in this country, and which certainly could not be taken about on field trips. The term »standard condition» must not be too rigidly interpreted if we are to arrive at a simple and generally practicable method. It should be born in mind also, that on account of the great individual variability which is characteristic of biological phenomena, the statistical mean of several approximate values renders better service in most cases than a few really exact determinations.

According to this general view, the following experimental procedure was adopted in the present investigation:

(a) Selection of plants

The majority of the experiments was performed in a cottage near Haugseter, Kvam, in the mountain district south of Rondane in Central Norway (2900 feet above sea level). The plant material was taken from the immediate vicinity of the cottage, which is situated on the tree-line. In the present paper only measurements in *Vaccinium myrtillus* L. are discussed. The blueberry was chosen as the first test object for several reasons: it is a very common plant in the district around Haugseter, it occurs in localities of rather different ecological character, and it responds to the different conditions of the habitat by marked morphological variation. Further, in most localities, a great number of individual plants are available, so that relatively homogeneous material can be secured for parallel or subsequent experiments. Owing to the small size of the leaves and the relatively slender stems, representative samples can be obtained within the weight range of the torsion balance employed (500 mg). The transpiration rates which can be observed in such

samples are, under ordinary conditions, of an order of magnitude favourable to determination by the rapid weighing method.

It deserves to be mentioned also, that other species of the same genus, but with different ecological properties, are available within the same area for comparative studies (*Vaccinium vitis-idaea* and *Vaccinium uliginosum*). Finally, the blueberry plants are practically unaffected by the heavy grazing which takes place during the entire summer season in this district.

For each experiment, healthy looking and habitually homogeneous plants were selected, usually among the individuals which seemed to be typical of the locality in question, i.e. of medium appearance in every respect. Only in ecologically extreme localities, plants close to the outside limits of the morphological variation range were picked out, in order that the possible influence of structural differences upon transpiration might be fully registered.

(b) *Pretreatment of the plant material*

Different methods of pretreatment were tried, apparently with equally good results. In every case, an ample number of plants was selected in the afternoon of the day before the experiment. In some cases, the plants were allowed to remain rooted over night, after being watered at about 19 o'clock, loosely packed with moist Sphagnum around the base, and covered by inverted glass jars, which were kept in place by small wooden rods driven into the ground. The plants were shielded against direct sunlight in the early hours of the morning by small gauze screens placed in front of the jars.

About 7 o'clock on the following morning the plants were cut at the base with a razor, immediately placed with the stems in water in a moist chamber, and transported to the cottage which served as a field laboratory. There the material was sorted and the stems of the selected objects cut once more, this time obliquely, under water, and several cm's above the previous cut.

Usually only sterile shoots were used, and where a few green, hard berries were occasionally found, they were removed. The objects were then deposited in moist chambers (loosely covered glass jars) with their cut stems in water, and the jars placed in a well lighted spot out-of-doors until the start of the experiment, care being taken to avoid direct sunlight.

In later experiments it was found to be more convenient to cut the plants at once on the evening preceding the experiment and place them in moist chambers over night. The objects were then always cut once more under water in the morning, a couple of hours before the start of the experiment. In periods of very cool weather, it was found necessary to transfer the plants to the weighing room some time before the first weighing, in order that they might assume room temperature before the experiment was started.

In the majority of the experiments, the stem was cut transversely and smeared with vaseline just before the first weighing. If necessary, an additional part of the stem was cut off in order to bring the weight of the object within the range of the balance. The basal part of the object was dried with filter paper, and small water droplets which might be attached to the leaves were carefully removed. Finally, each object was fitted with a small metal hook for hanging on the balance. The additional weight of the hook (10 mg) was compensated by means of the zero adjustment of the balance.

Table 2. *Results of experiment 2.* For photoperiodic treatment see figure 2.

Group	Dissected and sectioned	Allowed to develop	Flowering	Vegetative	Unclassified
A	7	1	7	0	1
B	2	6	0	8	0
C	3	5	2	3	3
D	2	6	8	0	0
E	3	5	8	0	0
F	2	6	0	8	0

period is interrupted 7—8 hours after its beginning or 6 hours from its end the plants remain vegetative. The effect of a light break about 14 hours after the beginning of the dark period is not clear (Group C) but a light break near the middle of the 38 hour dark period permits or even promotes flowering. The flowering response of group D was noticeably greater than that of groups A or E.

Photoperiodic processes occurring in the main light period are considerably enhanced in higher light intensities (Mann, 1940; Fabian, 1938). In the next experiment summer daylight was used to provide the main light period instead of the lower intensities available in the growth cabinet.

Experiment 3. 94 plants were used, in 7 groups of 10 and 2 of 12. These groups received photoperiodic treatments as shown in figure 3.

General conditions:

Main light period: 8 hours (9.0 a.m. to 5.0 p.m.), daylight, temperature not controlled.

Dark period: Temperature: $20^{\circ}\text{C} \pm 1$.

Light breaks: $\frac{1}{2}$ hour at 80 ft. candles, filament lamp.

Duration of treatments: 24 days.

Treatments were discontinued when the abscission of leaves threatened to endanger the plants; the degree of abscission was greatest in Group F. After 13 days (in excess of 16 hours daylight per day) following the photoperiodic treatment the plants were re-examined and the results shown in Table 3 obtained.

Apart from the higher light intensity and shorter duration of the photoperiod the treatments used were similar to those in Expt. 2, with the addition of groups C, H and J, in each of which the plants received two light breaks during the dark period. All the treatments resulted in a considerable shedding of mature leaves, but those plants which eventually flowered suffered more than those which remained vegetative. Flowering was postponed by a light break at 10 hours from the beginning of the dark period or 6 hours from its end. Light breaks interpolated at 16, 22 or 28 hours from the beginning

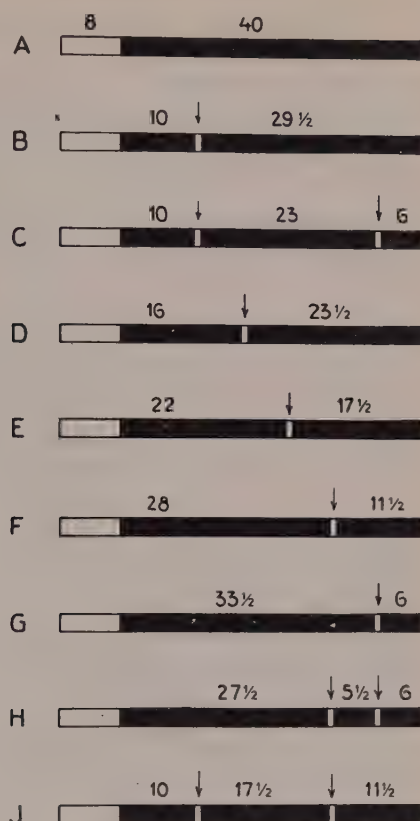


Figure 3. Photoperiodic treatment in experiment 3. Cf. legend to figure 1.

of the dark period had no inhibitory effect on flowering. Combinations of two light breaks were most effective in preventing flowering when given as in group C, 10 hours from the beginning and 6 hours from the end of the dark period. Group H received a combination of the treatments of groups

Table 3. Results of experiment 3. For photoperiodic treatment see figure 3.

Group	Flowering	Vegetative	Not classified	Total
A	11	0	1 (dead)	12
B	1	9	0	10
C	0	10	0	10
D	9	1	0	10
E	10	0	0	10
F	9	0	1 (dead)	10
G	2	8	0	10
H	3	7	0	10
J	2	10	0	12

III. Theory of transpiration decline in excised plants

The object of previous transpiration experiments with excised plants has mostly been to obtain instantaneous determinations of transpiration under natural conditions. In general, therefore, only a few weighings have been made during the first minutes after cutting. The first attempt at analyzing the subsequent course of transpiration during the following hours was made by Fukuda (1935), using the available experimental data of Schratz (1931 a, 1932), Pfleiderer (1933) and others.

The theory outlined by Fukuda has, however, in my opinion not received the attention which it deserves. This may be due partly to the somewhat confusing manner in which the theory was presented in the original paper, where rather remotely connected items were thrown together in a discussion of Walter's »hydrature»-conception, a discussion which, moreover, was not free from serious misunderstandings, as pointed out by Walter himself (1936). It seems to me, however, that Walter in his justified criticism has failed to grasp the essential point in Fukuda's way of thinking, nor has it been clearly understood in later literature, as far as I have been able to ascertain. It seems, therefore, that a more detailed treatment of the problem at this place may be justified. In the following, I shall try to develop the theory, as I understand it, independently of the »hydrature»-conception, in a way which may allow the underlying presumptions to emerge more clearly than perhaps they did in Fukuda's paper. Further, the practical application of the theory to experimental results will be dealt with at some length, and a new method of calculation introduced. As I do not think it worth while to state separately every point where the exposition given here is at variance with Fukuda's original formulation, I shall confine myself to a general reference to his paper.

The general idea of Fukuda's theory has already been indicated above (p. 65), but a few supplementary remarks may be required before we enter upon mathematical formulations. The fact that the transpiration rate of excised objects under constant conditions decreases with time, may be supposed to be due primarily to two causes, viz., decreasing water content and closing of stomata. If the aperture of stomata remained constant for some time during an experiment, and if no other changes interfered with transpiration, the decrease in the rate of water loss observed during this period ought therefore to be directly related to the decreasing water content (increasing water deficit) of the object. Suppose this relation could be quantitatively expressed by a simple formula, then a deviation from the theoretical curve derived from this formula, would indicate a change in stomatal aperture. Hence, it might be possible to obtain evidence of stomatal movements from the transpiration curve.

Table 4. *Results of experiment 4.* For photoperiodic treatment see figure 4.

Group	Mean score per plant ^a	S.D.*
A	26.6	11.31
B	16.0	14.67
C	0.875	1.25
D	10.4	9.15

^a Standard deviation of the mean.

used to compare the effects of various treatments on plant development. In the present instance, in order to indicate the wide range of variability the standard deviations of the scores within each group have been calculated, but statistical comparison between groups is probably invalid owing to the difficulty of weighting. (Table 4).

The results agree with those obtained in the experiments on *Perilla*. The inhibitory effect of the two light breaks given in group C is very striking.

Experiment 5. It was considered that a longer light break than that used in Expt. 4 might reduce the variability of the results, and in this experiment the light breaks lasted one hour at 50 ft. candles. It was also necessary to investigate the effectiveness of such a light period as an inductive photoperiod, acting in conjunction with a long dark period (in excess of 16 hours). One of the five groups of plants was therefore first subjected to the »short cycle» treatment devised by Hamner. This consists of a number of cycles each of 3 minutes light and 3 hours of darkness. This treatment effaces the influence of previous regimes of light and darkness on *Xanthium*. After receiving 12 »short cycles» the plants (group E) were exposed for one hour to light of 50 ft. candles (measured at the tops of the plants) provided by a daylight-type fluorescent lamp. They were then returned to dark cupboards for 23 hours, after which they were again subjected to the same light treatment (1 hour, 50 ft. candles). This procedure was repeated so that in all 3 cycles of 1 hour of light followed by 23 hours of darkness were received by these plants, which were then returned to long-day conditions.

The treatment in this and the other groups is represented in diagram 5.

General conditions:

Main light period: 12 hours, daylight (sunlight), temperature not controlled.

Dark period: 20° C \pm 1.

Light breaks: 1 hour, 50 ft. candles, daylight-type fluorescent lamp.

Each group received three cycles of treatment, and was then returned to long day conditions. After 24 days the terminal buds were dissected and the following measurements were taken:

- (a) Diameter of terminal inflorescence in mm.
- (b) Number of female flowers developed.
- (c) Stage of development of the female flowers.

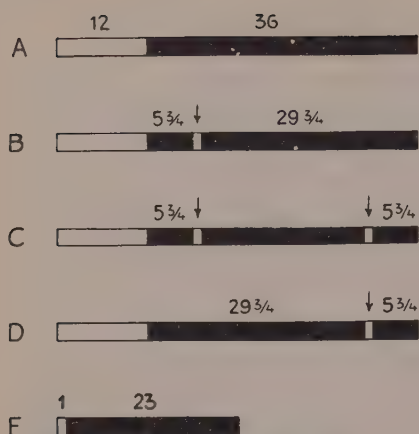


Figure 5. *Photoperiodic treatment in experiment 5. Cf. legend to figure 1.*

Correction: All $\frac{3}{4}$ should read $\frac{1}{2}$.

For the stages of development of the female flowers the following method of scoring was used:

	Score
Enlargement of apex of flower primordium	$\frac{1}{2}$
Flattening of apex	1
Formation of »ring-wall» of ovary	2
Closure of ring-wall	3
Development of spines	4

The variability within groups was considerably less than in Expt. 4. The results are given in Table 5.

It is evident from the very low score of plants of group E that one hour of light of 50 ft. candles is insufficient to constitute a photoperiod (when followed by 23 hours of darkness) even though this treatment is repeated three times. Therefore the light breaks in the other groups cannot constitute effective photoperiods in themselves. Apart from the rather higher score of group C the other results are effectively the same as in Expt. 4.

Table 5. *Results of experiment 5. For photoperiodic treatment see figure 5.*

Group	Mean score per plant
A	17.0
B	7.36
C	6.76
D	9.86
E	0.93

Discussion

The reactions in the leaves generated by cyclic alternations of periods of light and darkness only lead to the onset of flowering in short-day plants if the light in the (main) light period or photoperiod is of a sufficient intensity and duration, and if the period of darkness exceeds a minimum length. With certain plants (e.g. *Xanthium*) the light period may be of indefinite length, but generally there is an upper limit to the length of the light period which will constitute a photoperiod. There is a well-defined minimum length of the dark period (8 $\frac{1}{2}$ hours with *Xanthium*) and periods of darkness shorter than this *critical dark period* are unfavourable to flowering. Short-day plants are very sensitive to interruption of the dark period by even very short periods of light of low intensity.

Hamner (1942) regarded the effect of light in the main light period as causing the formation in the leaf of a hypothetical condition or substance »A», and in short-day plants the effect of a light break is to break down a photolabile condition or substance, »B», formed in the leaf in the dark. In the absence of a light break the amount of B increases until a state is reached where the amounts of A and B are such as to allow the formation of a flower inducing substance C.

For various reasons Gregory (1948) has incorporated this »three substance» scheme into a more precisely defined theory, according to which the substance B is produced in the dark from A, the light-formed substance. The transformation is reversible under the action of light so that a light break is regarded as effecting a photochemical back-reaction $B \leftarrow A$. The rate of formation of B increases during the dark period so that »increasing inhibition by the light break is a measure of the rate of formation of B» within the leaf. While B is being formed it is translocated to the meristems. The rate or amount of translocation is initially small but, after the maximal concentration of B has been attained, it becomes large and the content of B in the leaf rapidly falls. Therefore the effectiveness of a light break rapidly declines towards the end of a critical dark period. As soon as it is out of the leaf B is converted to the photostable flower inducing substance, C.

On Gregory's theory the effect of a light break is governed by two considerations: — (1) the amount of photolabile substance B present in the leaf at the time the light break is given: (2) the duration of the period of darkness following the light break, since, given sufficient time, the A produced photochemically from B during the light break will be re-converted into B, which will then be translocated and stabilised. A light break given near the middle of a critical dark period has a maximal inhibitory effect on flowering because (1) it encounters a maximal amount of B in the leaf: (2)

the ensuing dark period is too short to allow the re-formation and translocation of an adequate amount of B. Before this point of maximum effectiveness, less B is encountered and more time for reconversion allowed, and after this point less B is acted upon by the light break. Consequently a light break before or after the point of maximal effectiveness has progressively less effect the nearer it is to the beginning or the end of the critical dark period.

When, however, the light break is followed by a dark period of more than critical duration time is allowed for the re-formation of B and its translocation to the meristems. It is difficult therefore to see in Gregory's theory any explanation of the inhibitory effect of light breaks followed, as in the experiments described above, by more than 24 hours of darkness.

After a dark period which exceeds the critical in duration the photolabile substance B is absent from the leaf and there should be no further sensitivity to a light break. This is not in accord with the experimental results. In both *Perilla* and *Xanthium* there is an inhibitory effect of a light break towards the end of a very long dark period. This is shown not only when a light break is given at that time alone, but also when given in conjunction with a light break near the beginning of the very long dark period: the combined effect is greater than the effect of either light break taken singly.

It might well be assumed that a light break in the middle of the long dark period acts as a very short photoperiod in conjunction with the remainder of the dark period. That this is not so is demonstrated in these experiments. In previous experiments (not reported here) with the same strain of *Perilla* it has been found that an hour of light of intensity about 1,200 ft. candles does not constitute a photoperiod. Since the light breaks used in the *Perilla* experiments were always less than this in duration and intensity they cannot have constituted effective photoperiods. In experiment 5 it is also shown that the duration and intensity of light used in the light breaks with *Xanthium* do not constitute photoperiods. The non-inhibitory or even somewhat favourable effects of a light break in the middle of the very long dark periods in the *Perilla* experiments cannot be due to its acting as a photoperiod.

Bünning has put forward (1937, 1944 a, 1944 b, 1946, 1948, 1950, 1951) a generalised scheme intended to comprehend the results so far known of the formative and other effects of different regimes of light and darkness on plants. As there is no account of this theory available in English a brief and therefore simplified resumé of it will be given here; a critical discussion of the theory is to be found in Melchers and Lang, 1948. The theory does not invoke specific formative substances but stresses rather the existence of different modes of activity during the periods of light and darkness. Bünning was led to the theory by a study of diurnal leaf movements; he was able to demonstrate the existence of an endogenous component of these movements

(1931, 1932, 1936). These diurnal movements are thus the resultant of (1) the impressed rhythm of external changes, e.g. of light and darkness: (2) an internal regulation, with a periodicity peculiar to each plant species. The rhythm of leaf movement may be compared with the swing of a pendulum, which requires an initial impulse (change from light to darkness or vice versa) but proceeds for a time with a periodicity dependent on the physical characteristics of the pendulum (species of plant) and on the temperature. The time of swing may be to some extent forced by a timed train of impulses (leaf movement regulated by an impressed rhythm of light and darkness, within certain limits). Outside these limits the rhythm reverts to the natural rhythm of the pendulum (endogenous rhythm of the plant). Oscillations will eventually cease in the absence of a further impulse (leaf movements eventually cease in complete darkness or light and are resumed when the lighting conditions are changed).

Short-day plants were found to differ from long-day plants in respect to these leaf-movements: the leaves of short-day plants rise either before or at the time onset of light, and commence to fall after about 12 hours. The leaves of long day plants begin to rise about 12 hours after the onset of light and fall after about 12 hours. Bünning concluded that light is favourable to flowering during the »rising» or *photophile* phase of the leaf movement, and inhibitory during the falling or *scotophile* phase. The inhibitory effect is well marked with short-day plants and less conspicuous or absent in long-day plants. Maximum sensitivity to light coincides with the middle of both photophile and scotophile phases in short day plants.

Although Bünning's theory originated in studies on leaf-movement, too much stress should not be laid on the characters of leaf-movements when considering the formative effects of photoperiodic treatments (Bünning 1950). Rather should the course of the photophile and scotophile phases be followed from the formative effects of light during these phases. The leaf-movements are merely convenient outward indications of internal changes in the activity of a plant and may not always be in step with or exactly represent these changes. Studies on the leaf-movements of *Perilla* have confirmed Bünning's data on this plant (1944 b), but the leaf-movements of *Xanthium* have not been recorded. Claes and Lang were unable to detect a continuation of leaf movements of *Hyoscyamus* into long dark periods, but this does not imply an absence of scotophile and photophile phases of sensitivity to a light break. Bünning has been able to interpret the results of a large number of photoperiodic experiments, including some not explained on other theories. The most conspicuous success of his theory is its explanation of the results of the experiment of Claes and Lang mentioned in the introduction to this paper. One of the requirements of the theory is that there should be a continuation

of the succession of the internal scotophile and photophile phases even in complete darkness following an alternation of darkness and light. That is, a photoperiodically sensitive plant should show periodic alternations in sensitivity to a light break during a very long dark period. A light break applied during the time when the internal activity of the plant is in its photophile phase should favour flowering, and conversely a light break during the scotophile phase should inhibit flowering. Now the photophile phase of a long-day plant such as *Hyoscyamus niger* begins (according to Bünning) about 10—12 hours after the onset of light and should last about 12 hours. During this phase the effectiveness of a light break in causing flowering should rise to a maximum and then fall to zero. This is of course in accordance with well-known facts and is explained on other theories than that of Bünning.

Let us consider however a period of 12 hours of light followed by a 36 hour dark period. The first 12 hours of darkness will coincide with a photophile period (with *Hyoscyamus*). In the next 12 hours the plant will be in its scotophile phase and light should have less or no effect on flowering. Finally the last 12 hours will coincide with a further photophile phase during which light will again favour flowering. *There will be a second rise in sensitivity during a 36 hour dark period.* These predictions are fulfilled in Claes and Lang's experiment; their results show that the flower-inducing effect of a light break rises to a maximum some 16 hours after the beginning of the main light period and falls to zero eight hours after reaching that maximum. Then in the last 12 hours of the dark period there is a similar rise and a slight decline towards the end. Claes and Lang did not detect a positive inhibitory effect during the middle of the long dark period since only degrees of flowering were estimated and not degrees of »vegetativeness».

In the experiments reported above essentially similar results were obtained with *Perilla* and *Xanthium*, but in an opposite sense, since these are short day plants. In the *Perilla* experiments it is shown that there is a qualitative difference between the effects of a light break towards either end of the long dark period and near the middle. These differences are in accord with those expected on Bünning's theory, as also is the summation of the inhibitory effects of interruption of both scotophile phases which will lead, as in the *Xanthium* experiments, to greater inhibition of flowering than interruption of either scotophile phase alone. One difficulty is that this explanation ignores the shifting of the endogenous rhythm of the plant caused by the light breaks themselves. The results are however sufficiently clear to warrant further investigation of the details of the reactions involved.

There is a salient point of difference between Claes and Lang's experiment and those reported above; in their experiment the light interruptions were relatively long

(2 hours) and of high light intensity. They can hardly be termed »light breaks» in the normal sense. Indeed, they might be regarded as very short main light periods, less of course in duration than the critical daylength for *Hyoscyamus*, but having effects possibly different from those of a light break of short duration and low intensity.

Claes and Lang have pointed out an alternative explanation of their results; a light interruption near the beginning of the dark period acts in conjunction with the previous main light period, and an interruption near the end of the dark period acts with the main light period which follows. They show that a critical experiment could be devised using a 72 hour cycle with a dark period during which three scotophile phases should occur. If then light in the second of these phases had the same effect as in the first or third, Bünning's theory would be supported, if not, the alternative explanation of Claes and Lang. Unfortunately this type of experiment has not been carried out, and it is likely that the mortality of plants under such conditions would be high. Moreover, no information appears to be available which would show that a short light break of low intensity can, in short-day plants, act in conjunction with a subsequent main light period.

Summary

The short-day plants *Perilla ocymoides* and *Xanthium pennsylvanicum* were subjected to photoperiodic cycles of 48 hours duration, each consisting of an 8, 10, or 12 hour photoperiod, followed by a long dark period. Light breaks were interpolated at various intervals during the long dark periods.

Flowering was inhibited by a light break near the beginning or end but not near the middle of the long dark period.

The results are compared with those of Claes and Lang on *Hyoscyamus niger*, a long-day plant, and are discussed in terms of various theories of photoperiodism.

The experimental data are shown to support Bünning's theory, but not that of Gregory. They may also be explained, as Claes and Lang suggest, if a light break can act in conjunction with a subsequent photoperiod.

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References

- Bünning, E.: Über die autonomen tagesperiodischen Bewegungen der Primärblätter von *Phaseolus multiflorus*. — Jahrb. wiss. Bot. 75: 439. 1931.
— Über die Erbllichkeit der Tagesperiodizität bei den Phaseolus-Blättern. — Jahrb. wiss. Bot. 77: 283. 1932.

- Die Entstehung der Variationsbewegungen bei den Pflanzen. — *Ergebnisse Biol.* 13: 235. 1936.
- Die endonome Tagesrhythmik als Grundlage der photoperiodischen Reaktion. — *Ber. deutsch. bot. Ges.* 54: 590. 1937.
- Endonome Tagesrhythmik und Photoperiodismus bei Kurztagpflanzen. — *Biol. Zbl.* 64: 161. 1944 a.
- Die allgemeinen Grundlagen der photoperiodischen Empfindlichkeit. — *Flora* 38: 93. 1944 b.
- Die entwicklungsphysiologische Bedeutung der endonomen Tagesrhythmik bei den Pflanzen. — *Naturwiss.* 33: 271. 1946.
- Weitere Versuche über die Beziehung zwischen endogener Tagesrhythmik und Photoperiodismus. — *Zeit. f. Naturf.* 3 b: 457. 1948.
- Über die photophile und skotophile Phase der endogenen Tagesrhythmik. — *Planta* 38: 521. 1950.
- Der tagesperiodische Verlauf der Substanzproduktion und speziell der Zellulose-synthese in Beziehung zur photoperiodischen Reaktionsweise. — *Planta* 39: 460. 1951.
- Über Langtagpflanzen mit doppelter photophiler Phase. — *Ber. deutsch. bot. Ges.* 54: 85. 1951.
- Claes, H. & Lang, A.: Die Blütenbildung von *Hyoscyamus niger* in 48-stündiger Licht-Dunkel-Zyklen und in Zyklen mit aufgeteilter Lichtphasen. — *Zeit. f. Naturf.* 2 b: 56. 1947.
- Fabian, I.: Beiträge zum Lang- und Kurztagsproblem. — *Zeit. f. Bot.* 33: 305. 1939.
- Gregory, F. G.: The control of flowering in plants. — *Symp. Soc. Exp. Biol. (Cambr.)* II: Growth, p. 75. 1948.
- Hamner, K. C.: Interrelation of light and darkness in photoperiodic induction. — *Bot. Gaz.* 101: 658. 1940.
- Hormones and Photoperiodism. — *Cold Spring Harbor Symp.* 10: 49—60. 1942.
- & Bonner, J.: Photoperiodism in relation to hormones as factors in floral initiation and development. — *Bot. Gaz.* 100: 388. 1938.
- Harder, R. & Bode, O.: Über die Wirkung von Zwischenbelichtungen während der Dunkelperiode auf das Blühen, die Verlaubung und die Blattsukkulenz bei der Kurztagspflanze *Kalanchoë Blossfeldiana*. — *Planta* 33: 469. 1943.
- Lang, A. & Melchers, G.: Die photoperiodische Reaktion von *Hyoscyamus niger*. — *Planta* 33: 653. 1943.
- Mann, L. K.: Some effects of environmental factors on floral initiation in *Xanthium*. — *Bot. Gaz.* 102: 339. 1940.
- Melchers, G. & Lang, A.: Die Physiologie der Blütenbildung. — *Biol. Zbl.* 67: 105. 1948.
- Naylor, A. W.: Effect of length of induction period on floral development of *Xanthium pennsylvanicum*. — *Bot. Gaz.* 103: 146. 1941.
- Parker, M. W., Hendricks, S. B., Borthwick, H. A. & Scully, N. J.: Action spectrum for the photoperiodic control of floral initiation of short-day plants. — *Bot. Gaz.* 108: 1. 1946.
- Wangermann, E. & Ashby, E.: Studies in the morphogenesis of leaves VII Pt. 1. Effects of light intensity and temperature on the cycle of ageing and rejuvenation in the vegetative life-history of *Lemna minor*. — *New Phyt.* 50: 186. 1951.

Chelidonic Acid and its Effects on Plant Growth

By

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Introduction

Chelidonic acid, a pyrone-ringed compound of the structure shown in Figure 1, is a naturally occurring compound, common in many plants and in some cases at quite high concentrations (Ramstad, in prep). The possibility that such a widespread compound might influence growth deserves investigation.

Naturally occurring plant constituents which inhibit growth have been found in several species of plants. Some striking examples of inhibitors have been found to be excreted from plant roots. These inhibitors can prevent seedlings of some species from growing nearby. For example, roots of guayule excrete trans-cinnammic acid which inhibits growth of guayule seedlings (Bonner, 1946); roots of walnut contain an inhibitor probably identical with juglone which is toxic to other seedlings (Davis, 1928). Leaves of *Encelia farinosa* give off a substance toxic to some seedlings (Gray and Bonner, 1948).

Unsaturated lactones have been shown to be strongly inhibitory of seed germination and of growth of stem sections as well (Veldstra and Havinga, 1945). One of these, scopoletin, has been found to inhibit the growth of oat roots at concentrations in which it occurs naturally in those roots (Goodwin and Taves, 1950).

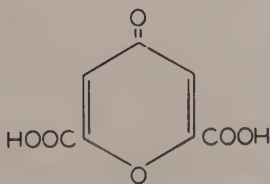


Figure 1. The structure of chelidonic acid.

The data presented below demonstrate that chelidonic acid is a naturally occurring growth inhibitor in plants.

Experimental

In order to measure the effect of chelidonic acid on growth, the standard pea straight growth test was used, as described by Went and Thimann (1937). It was found that chelidonic acid inhibits growth at concentrations as low as 0.1×10^{-4} M. Figure 2 shows the type of inhibition curve obtained. A 50 per cent inhibition of growth is obtained at concentrations of about 10^{-4} M. No significant promotion of growth has been obtained with chelidonic acid alone, but only inhibitions. Hence it appears that chelidonic acid is an inhibitor of growth, and since it apparently cannot promote growth by itself at low concentrations, it is not a growth regulator in the same sense as auxins are.

In an effort to examine more closely the type of inhibition of growth exerted by chelidonic acid, pea straight growth tests were carried out with auxin (indole acetic acid, IAA) added as well as the inhibitor. As can be seen from

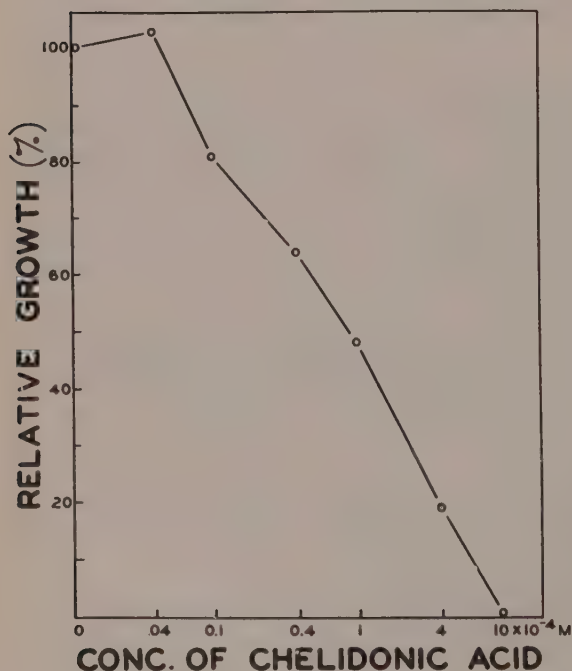


Figure 2. *The inhibitory effect of chelidonic acid on growth.* (Pea straight growth test, buffered at pH 5.0). Least significant difference at 1 % level, 30 %. Growth of controls = 2.11 mm.

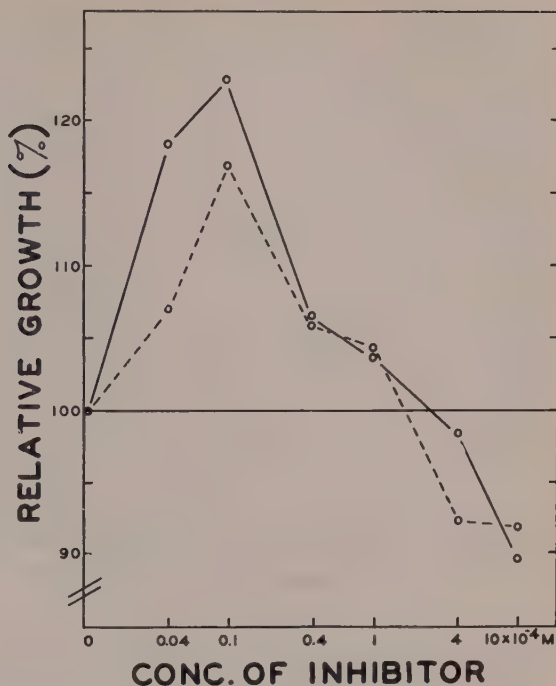


Figure 3. The effect of chelidonic acid (dashed line) and coumarin (solid line) on growth in the presence of 0.1 mg/l indoleacetic acid. (Pea straight growth test). Least significant difference at 1 % level, 10.9 %. Growth of controls = 4.4 mm.

Figure 3, in the presence of 0.1 mg/l of IAA the compound acts in quite a different way: the inhibition effect was observed only at much higher concentrations (4×10^{-4} M and higher), and in the lower concentrations a very marked promotion of growth prevailed. Under the conditions of these tests, maximum promotion (17 %) occurs at 0.1×10^{-4} M. This promotive effect in the presence of auxin has been confirmed in slit pea tests, straight growth tests, and in the *Avena* test.

Among the many chemicals which will inhibit growth, there are some which in their behavior are strikingly similar to chelidonic acid. For example, unsaturated lactones are known to inhibit growth, and in the presence of auxin they too will promote growth. Thimann and Bonner (1949) have demonstrated that coumarin in the presence of 1 mg/l IAA promotes growth 20 per cent at 10^{-5} M, and inhibits growth 50 per cent at 10^{-3} M. A direct comparison of the synergistic activities of coumarin and chelidonic acid determined simultaneously can be seen in Figure 3.

The inhibitory action of the lactones with which Thimann and Bonner worked was relieved by the sulfhydryl-containing compound, 2, 3 dimercapto-1-propanol (BAL). They deduce that the inhibition of growth by unsaturated lactones is a consequence of their reactivity with sulfhydryl groups. The addi-

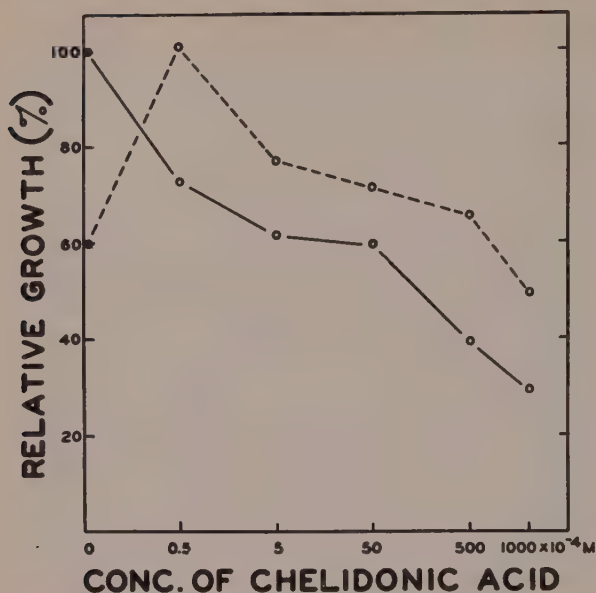


Figure 4. *Reversal of the chelidonic acid effect by BAL ($10^{-4} M$). Solid lines: Chelidonic acid plus 0.1 mg/l IAA. Dashed lines: Ditto after presoak in $10^{-4} M$ BAL. (Pea straight growth test). Least significant difference at 1 % level, 16.3 %. Growth of controls = 3.8 mm.*

tion of more sulfhydryl groups in the form of BAL is presumed to act in a protective way toward enzyme sulfhydryl groups in the cells.

The similarity of the growth behavior of chelidonic acid to the unsaturated lactone, coumarin, and its similarity in form to the structure of a typical γ -lactone suggests the possibility that chelidonic acid may operate against growth by the same means as unsaturated lactones. To test this possibility, tests were carried out in which BAL was added as a pre-soak treatment before the application of the auxin and chelidonic acid. The results of such a test are shown in Figure 4. It can be seen from these data that the effect of the BAL was to relieve quantitatively the chelidonic acid effect at every concentration from 0.5 to $1000 \times 10^{-4} M$.

Discussion

The fact that free chelidonic acid occurs in large quantities in many plants makes it a particularly interesting inhibitor of growth. The possibility that it, and perhaps compounds which are closely related structurally, might influence normal plant development and growth seems very likely indeed. Chelidonic acid has been found by Ramstad (in prep.) to occur in several species in concentrations as high as $10^{-2} M$, calculated on a wet weight basis. This is 1,000 times more concentrated than that needed for inhibition of growth in the pea straight growth test.

It has been suggested by Thimann (1951) that the mechanism of auxin action in plants may be through a protection against natural inhibitors of some stages of organic acid metabolism. He pointed to its action in preventing inhibition by such sulfhydryl inhibitors as iodoacetate as evidence for his suggestion. The evidence obtained in this study strongly supports such an hypothesis, for in the absence of auxin, chelidonic acid was found to be a strong inhibitor of growth, while in the presence of auxin the inhibition is entirely lost over a wide range in concentrations (cf. Figures 2 and 3).

The action of chelidonic acid in inhibiting growth shows some striking parallels to the action of unsaturated lactones such as coumarin. Both of these compounds are themselves inhibitors of growth, and in the presence of auxin they show a synergistic behavior in that they can increase growth in the presence of small concentrations of auxin. A second parallel between the two is found in the relief of the growth effects by the sulfhydryl compound BAL. It would appear likely then that chelidonic acid may have its effect on growth in approximately the same way as coumarin, which is thought to be due to its reactivity with sulfhydryl positions on enzymes involved in growth.

The common unsaturated lactones, such as coumarin and most of its substituted derivatives, have not been found to occur in considerable quantity in the free state. Indeed, most of them apparently exist in plants as components of plant glycosides. An exception appears to be the case of scopoletin. This compound has been found to be present in *Avena* roots in concentrations of about 5×10^{-5} M, at which concentrations it begins to inhibit growth of these roots. Chelidonic acid on the other hand commonly occurs in plants in the free state at concentrations as much as one thousand times greater than that required for initial inhibition of growth.

Summary

The action of chelidonic acid in growth has been studied by means of the pea straight growth test. The following conclusions have been drawn:

1. Chelidonic acid is a naturally occurring inhibitor of growth in plants.
2. It is capable of inhibiting growth at concentrations of 10^{-5} M, a concentration 1,000 times more dilute than it sometimes occurs naturally.
3. Its inhibitory action can not only be relieved, but actually be changed into a promotive action by the presence of auxin. At 10^{-5} M, growth is increased 17 per cent in the presence of 0.1 mg/l indole acetic acid and inhibition effects are first found at 4×10^{-4} M. This type of behavior is

characteristic of synergists of auxin such as the unsaturated lactone, coumarin.

4. The inhibitory effect of chelidonic acid can be relieved by the sulfhydryl compound 2,3 dimercapto-1-propanol (BAL). It is suggested that chelidonic acid may act in growth by the same means as does coumarin.

References

1. Bonner, J.: Relation of toxic substances to growth of guayule in soil. — *Bot. Gaz.* 107: 343. 1946.
2. Davis, E. G.: Toxic principle of *Juglans nigra* as identified with juglone. — *Am. J. Bot.* 15: 620. 1928.
3. Goodwin, R. H. & Taves, C.: Effect of coumarin derivatives on the growth of *Avena* roots. — *Am. J. Bot.* 37: 224. 1950.
4. Gray, R. & Bonner, J.: An inhibitor of plant growth from leaves of *Encelia farinosa*. — *Am. J. Bot.* 35: 52. 1948.
5. Ramstad, E.: The occurrence and distribution of chelidonic acid in some plant families. — (In prep.).
6. Thimann, K. V.: The synthetic auxins: relation between structure and activity. — In Skoog, »Plant Growth Substances». University of Wisconsin Press, 1951.
7. Thimann, K. V. & Bonner, W. D.: Inhibition of plant growth by protoanemonin and coumarin, and its prevention by BAL. — *Proc. Natl. Acad. Sci.* 35: 272. 1949.
8. Veldstra, H. & Havinga, E.: Physiological activity of unsaturated lactones. — *Enzymologia*, 11: 373. 1945.
9. Went, F. & Thimann, K. V.: *Phytohormones*. — Macmillan Company, N. Y. 1937.

Maleic Hydrazide as an Anti-Auxin

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Maleic hydrazide has been reported to have a profound influence on plant development when applied as a plant spray. Schoene and Hoffmann (15) were first to observe that young tomato plants respond to this compound by a cessation in growth and loss of apical dominance. Subsequent investigations have demonstrated that maleic hydrazide can also prolong dormancy (4, 20), can cause the abscission of young flowers or fruits (10, 12, 19, 21) or can kill plants as an herbicide (3, 11, 21). These reports in addition to the observations that maleic hydrazide can be used to prevent flowering (6, 12) make this compound most interesting from a physiological point of view.

The means by which maleic hydrazide might have these diverse effects on plant growth are not yet clear. The first evidence as to how this compound might function in plants was proposed by Leopold and Klein (9), who demonstrated that maleic hydrazide can act as an anti-auxin in growth. The present study was undertaken to further clarify this action.

Methods and results

In studying the action of maleic hydrazide (MH) on growth, the standard pea and *Avena* growth tests described by Went and Thimann (18) have been used except where otherwise stated. These tests give a precise control of the factors involved in growth, and permit an effective approach to the study of the problem in the present investigation.

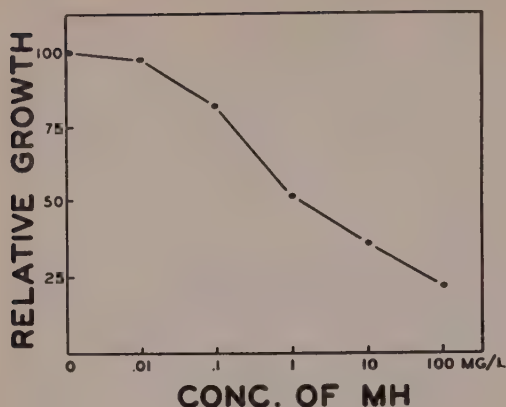


Figure 1. *Inhibition of growth by maleic hydrazide.* (Pea straight growth test.) Least significant difference at 1 % level, 12 %.

Maleic hydrazide as a growth inhibitor

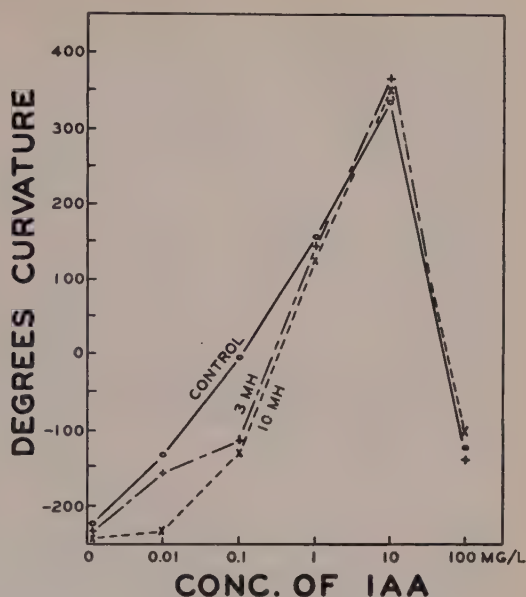
In their original publication, Schoene and Hoffman speak of MH as a »growth regulant» in view of the inhibitory effects on growth which they observed. This term implies that the compound is a growth regulator in the same sense as are auxins. True growth regulators of the auxin type all stimulate growth in low concentrations and inhibit growth at higher concentrations. Repeated attempts with the standard pea and *Avena* straight growth tests have failed to show any significant increase of growth when MH is applied at concentrations between 10^{-3} and 10^{-10} M. Instead, MH inhibits growth in approximately a semi-logarithmic manner. Results of a representative pea straight growth test are presented in figure 1. It can be seen from these data that MH inhibits growth at concentrations as low as 0.1 mg/l, or 9×10^{-7} M. The minimum concentration for inhibition of growth varies somewhat from one experiment to another, apparently due to differences in the initial rate of growth of the plant material used. However, inhibition by 0.1 mg/l is typical of most pea and *Avena* straight growth tests.

It is evident that MH is a growth inhibitor. Since no promotion of growth is obtained with MH alone, it is likewise evident that it is not a growth regulator of the auxin type. Thus, to call this growth inhibitor a »growth regulant» would seem misleading.

Maleic hydrazide as an anti-auxin

MH has been found by several workers to break apical dominance in plants (4, 6, 11, 13, 15). Since auxin is a primary controlling agent of apical dominance (18), the question arose as to whether the MH is acting in opposi-

Figure 2. *Inhibition of growth by maleic hydrazide (3 and 10 mg/l), and the relief of the inhibition by high levels of indoleacetic acid. (Slit pea test.)* Least significant difference at 1 % level, 57°.



tion to auxin. If this is the case, then it would be expected that a given inhibition by MH might be overcome by the addition of auxin.

As has been briefly reported elsewhere (9), auxin is capable of overcoming inhibitions by MH in the standard pea tests. A graphic demonstration of auxin reversal of MH inhibition in the slit pea test is shown in figure 2. In this test, 3 and 10 mg/l of MH were used to produce an initial inhibition of growth, and indoleacetic acid (IAA) was added in serial concentrations. It is evident from figure 2 that at concentrations of IAA less than 1 mg/l the curvature of stem sections is strongly inhibited by the presence of 3 or 10 mg/l of MH. However, at higher concentrations of IAA the inhibition disappears — that is, curvature in the presence of MH is no less than curvature without it. The ability of high concentrations of IAA to overcome MH inhibitions has been confirmed with the Avena and pea straight growth tests.

From the evidence presented thus far, it appears that MH is capable of inhibiting growth, and that the inhibition effect can be alleviated by IAA and can even be made to disappear by the presence of large amounts of IAA. If MH is acting in opposition to auxin, then it might be expected to alleviate the inhibition of growth resulting from very high concentrations of auxin. In other words, if MH reduces the effectiveness of auxin in growth, then when a lack of auxin is limiting growth, added MH should accentuate this limitation and reduce growth; however, when auxin is present in excess, and hence is limiting growth, added MH should mitigate this limitation and increase

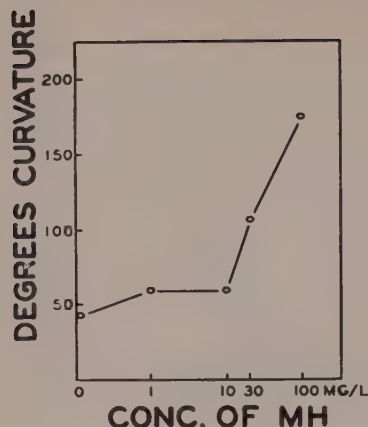


Figure 3. *Relief of indoleacetic acid inhibition of growth by high levels of maleic hydrazide. (Slit pea test.) All dishes contained 30 mg/l indoleacetic acid. Least significant difference at 1 % level, 35°.*

growth. In order to determine whether this increase in growth can be obtained, slit pea tests were carried out using superoptimal concentrations of IAA, and adding various quantities of MH. Results of such a test are presented in figure 3, from which it can be seen that the amount of curvature obtained with 30 mg/l IAA (an inhibitory auxin level, cf. figure 2) can be increased by the addition of large amounts of MH. Growth in the IAA alone was found to be only 42 degrees. With the addition of 100 mg/l of MH, growth was increased to 174 degrees, a gain of 132 degrees, which gain is fully significant at the 1 % level. The ability of MH to alleviate auxin inhibition has been confirmed using four different inhibitory levels of auxin.

The question arises as to the possibility that the capacity of MH to overcome auxin inhibition could conceivably be a pH effect. That is, if MH raises the pH of the auxin solution, it might effectively reduce the penetration of the auxin and hence appear to overcome auxin inhibition without actually doing so directly. The use of buffer solutions in the pea test requires such strongly buffered solutions in addition to the large inhibitory amounts of auxin plus the MH, that well buffered tests to study this question invariably result in flaccid plant material and unreliable results. Consequently a different test was turned to for this part of the study. By use of 10 mm. sections of pea roots instead of stems, positive growth responses can be obtained with very much smaller quantities of IAA, and maximum growth is obtained with 0.01 mg/l IAA. Thirty-five per cent inhibition of growth is obtained with 0.1 mg/l of IAA. A test was therefore carried out using an inhibitory concentration of auxin (0.5 mg/l), with MacIlvaine's buffer diluted 1: 8, at pH 5.0, and MH added in serial concentrations. At the conclusion of the test, all solutions showed a pH of 5.0 as indicated on a pH meter sensitive to differences of 0.1. The results are presented in table 1. It can be seen that the

Table 1. *Reversal of auxin inhibition of growth with maleic hydrazide. Pea root test, buffered at pH 5.0.*

Treatment	Growth	Increase Over Control
IAA (0.5 mg/l)	1.7 mm.	—
IAA+0.01 mg/l MH	2.0	+ 17 %
IAA+0.05 mg/l MH	2.2	+ 29 %
IAA+0.1 mg/l MH	3.4	+100 %

LSD at 1 % level; 5 %.

addition of 0.1 mg/l MH increased growth two-fold over the auxin control. This increase is fully significant at the 1 % level, and demonstrates that the capacity of MH to alleviate the auxin inhibition of growth is independent of pH.

Mechanism of the anti-auxin action

In view of the apparent mutual antagonism between IAA and MH, it would be interesting to know whether there might be a direct interaction or combination between the two compounds. In view of their structural characteristics, a reaction between the two would seem unlikely. However, to test the possibility, diffusion rate experiments similar to those of Gordon and Nieva (5) were carried out with IAA alone and in the presence of MH. If a reaction or bonding occurs between the two compounds, the resulting increase in molecular size should considerably alter the diffusion rate.

Discs of 1.5 per cent agar 1.42 mm. thick and with a volume of 0.26 ml. were arranged in replicated piles of four discs. One-half of the piles had 5×10^{-4} M MH. Diffusion was permitted for two hours at 25° C., after which time the amount of IAA in each block was determined by the Salkowsky reaction, using the techniques described by Tang and Bonner (16). Three

Table 2. *The distribution of indoleacetic acid in agar blocks after diffusion, in the absence and presence of maleic hydrazide. (Mean of three experiments)*

Block	Micrograms of IAA/ml.		Per Cent Distribution of IAA	
	IAA alone	IAA + MH	IAA alone	IAA + MH
1	19.0	19.6	34.0 %	35.2 %
2	15.8	15.3	28.2	27.4
3	11.9	11.6	21.2	20.9
4	9.3	9.2	16.6	16.5
Total IAA Recovered:	56.0	55.7	100.0 %	100.0 %

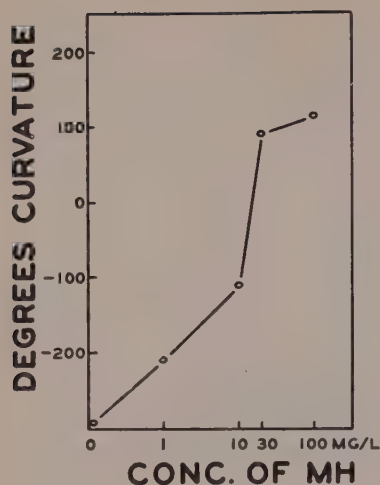


Figure 4. *Relief of naphthaleneacetic acid inhibition of growth by high levels of maleic hydrazide.* (Slit pea test.) All dishes contained 30 mg/l naphthaleneacetic acid. Least significant difference at 1% level, 75°.

homologous blocks were placed in each colorimeter courvette, and four ml. of acid reagent were added. The colorimeter readings were converted to absolute amounts of IAA by reference to a calibration curve previously established by the same technique. The mean distribution of the IAA in each of the four blocks of the pile is presented in table 2. From the data it can be seen that (1) there was no change in the distribution of IAA between blocks when MH was present, and (2) there was no change in the total amount of IAA accounted for when MH was present. The experiment demonstrates that the diffusion rate of IAA is unchanged by the presence of MH, and therefore, there probably was no reaction between the two compounds *in vitro* under the conditions of the experiment.

Further evidence that there is no direct reaction between IAA and MH was sought in two ways. (1) Slit pea tests were carried out in which the MH was added separately in a presoaking treatment, followed by IAA solutions one hour later. This was done to determine whether the two compounds were reacting before entering the pea section. The results obtained showed no significant differences between the MH presoaking and the MH in the auxin solution treatment. (2) Slit pea tests were carried out at an inhibitive level of naphthaleneacetic acid (30 mg/l), with serial concentrations of MH added, to determine whether MH is specific in its action against IAA only. The results obtained (figure 4) are entirely comparable to those obtained using IAA presented in figure 3. Since naphthaleneacetic acid is considerably more stable than IAA (18), it would seem even less likely that MH combines with auxins or growth regulators directly. In short, no evidence could be

obtained which would demonstrate that a reaction occurred between IAA and MH *in vitro*. Consequently the antagonism between the two compounds cannot be explained on that basis.

Discussion

Previous workers have observed that MH is capable of inhibiting growth of intact plants, and in doing so it has quite generally broken apical dominance. Since auxins promote growth, and control apical dominance, a compound which acts against auxin should be able to both inhibit growth and break apical dominance. The present study has demonstrated that an antagonism exists between maleic hydrazide and auxin in growth. This has been shown in two ways: First, the inhibition of growth resulting from low concentrations of MH has been overcome completely by the addition of auxin; and second, the inhibition resulting from excessive concentrations of auxin has been overcome by the addition of MH. To the authors' knowledge, this is the first report of any substance which is mutually antagonistic with auxin, i.e., each can relieve inhibition produced by the other.

Van Overbeek *et al* (14) have shown that trans-cinnamic acid inhibition of growth can be completely overcome with auxins, or growth regulators, and have used this information to compare growth regulator activities. Trans-cinnamic acid is structurally similar to growth regulators in that it has the two molecular constituents considered to be essential (7): viz. an unsaturated aromatic ring, plus an acid side-chain. However, due to the spatial position of the side-chain, it lacks growth regulator activity, and instead acts in opposition to growth regulators and auxins. Burström (2) has demonstrated anti-auxin properties in other auxin homologues, particularly ones with dimethyl substitutions in the acid side-chain. In the case of MH, however, there is no acid side-chain at all. Therefore, it is rather unlikely that it acts as an anti-auxin in the same way as the auxin homologues.

Åberg (1) has demonstrated some anti-auxin properties of two compounds, 1-naphthol and α -naphthylmethylsulfide propionic acid. The first of these is evidently not an auxin homologue. These substances were shown to be effective in relieving the inhibition of root growth by auxin. No information is available as to whether auxin can relieve an inhibition produced by these compounds.

The possibility that MH reduces the effectiveness of auxin in growth by directly combining with the IAA molecule *in vitro* appears unlikely in view of the fact that the diffusion rate of IAA is unchanged by the presence of MH, hence the molecular weight is unchanged. Further evidence that no

reaction occurs between the two is found in the fact that exposing the plant material to the two compounds separately and consecutively has the same effect as applying them together, and furthermore, the action of MH is not specific for IAA because it acts as effectively against naphthaleneacetic acid.

The flaccid condition of pea stem sections resulting from 100 mg/l of IAA is generally relieved by 10 mg/l of MH. This protection against auxin injury was evident in several tests in which large amounts of both of these compounds were used. Incidentally, this is in contrast to the effect of the anti-auxin trans-cinnamic acid reported by van Overbeek *et al* (14). They found that trans-cinnamic acid caused loss of turgescence at lower concentrations of auxin than did the auxin alone.

The ability of MH to act in opposition to auxin is highly compatible with the observations of its effects on intact plants. Its capacity for stopping stem elongation and leaf enlargement and for prolonging dormancy of buds and bulbs otherwise ready to germinate (4, 20, 21) may be interpreted as resulting from its opposition to auxin action; for auxin is essential in stem, leaf, and bud growth. (18). Its capacity for nullifying apical dominance likewise may be attributed to its opposition to auxin action, for auxin can inhibit lateral bud development (8, 17), and an auxin antagonist would logically be expected to break apical dominance. Maleic hydrazide has also been observed to inhibit flower initiation in several species (6, 12), and suggestions as to how this may be related to the anti-auxin characteristics demonstrated here are proposed by Klein and Leopold (6).

Summary

1. Using standard pea growth tests, the action of maleic hydrazide on growth has been investigated.
2. Maleic hydrazide was found to be a growth inhibitor. In the absence of auxin it inhibits growth at concentrations as low as 0.1 mg/l, or 9×10^{-7} M; 50 per cent inhibition is obtained with about 1.0 mg/l. Since it is apparently incapable of promoting growth in the absence of auxin, it is not a growth regulator.
3. Inhibition of growth by low concentrations of maleic hydrazide is completely relieved by the addition of auxin.
4. Conversely, inhibition of growth by high concentrations of auxin can be relieved by the addition of maleic hydrazide. Tests with pea roots show this reversal of auxin inhibition to be independent of any pH change.
5. No evidence could be found which would indicate that the inhibitor acts by directly combining with auxin *in vitro*.

6. It is concluded that maleic hydrazide is an anti-auxin, and acts in opposition to auxin in growth.

Grateful acknowledgement is made to Miss Frances I. Scott for her assistance in carrying out some of the tests.

References

1. Åberg, B.: On auxin antagonists and synergists in root growth. — *Physiol. Plant.* 3: 447. 1950.
2. Burström, H.: Studies on growth and metabolism of roots. VI. The relative growth action of different isobutyric acid derivatives. — *Physiol. Plant.* 3: 470. 1951.
3. Currier, H. B. & Crafts, A. S.: Maleic hydrazide, a selective herbicide. — *Science* 111: 152. 1950.
4. Fillmore, R. H.: Control of plant development with maleic hydrazide. — *Arnoldia* 10: 33. 1950.
5. Gordon, S. A. & Nieva, F. S.: Biosynthesis of auxin in the vegetative pineapple. I. — *Arch. Biochem.* 20: 356. 1949.
6. Klein, W. H. & Leopold, A. C.: Effect of maleic hydrazide on flower initiation. (In prep.)
7. Koepfli, J. B., Thimann, K. V., & Went, F. W.: Phytohormones: structure and physiological activity. — *Jour. Biol. Chem.* 122: 763. 1938.
8. Leopold, A. C.: The control of tillering in grasses by auxin. — *Am. J. Bot.* 36: 437. 1949.
9. Leopold, A. C. & Klein, W. H.: Maleic hydrazide as an anti-auxin in plants. — *Science* 114: 9. 1951.
10. Miller, E. R. & Erskine, D.: Prevention of fruit formation on some ornamental trees. — *Proc. 25th. Natl. Shade Tree. Conf., FP.* 88. 1949.
11. Moore, R. H.: Effects of maleic hydrazide on plants. — *Science* 112: 52. 1950.
12. Naylor, A. W.: Effects of maleic hydrazide on flowering of tobacco, maize and cocklebur. — *Proc. Natl. Acad. Sci.* 36: 230. 1950.
13. Naylor, A. W. & Davis, E. A.: Maleic hydrazide as a plant growth inhibitor. — *Bot. Gaz.* 112: 112. 1950.
14. van Overbeek, J., Blondeau, R., & Horne, V.: Trans-cinnamic acid as an anti-auxin. — *Am. J. Bot.* 38: 589. 1951.
15. Schoene, D. L. and Hoffmann, O. L.: Maleic hydrazide, a unique growth regulant. — *Science* 109: 588. 1949.
16. Tang, Y. W. & Bonner, J.: The enzymatic inactivation of indoleacetic acid. I. — *Arch. Bioch.* 13: 11. 1947.
17. Thimann, K. V. & Skoog, F.: Inhibition of bud development and other functions of growth substance in *Vicia Faba*. — *Proc. Roy. Soc. London Bllr.* 317. 1934.
18. Went, F. W. & Thimann, K. V.: *Phytohormones*. — MacMillan, New York. 1937.
19. White, D. C. & Kennard, W. C.: Preliminary report on the use of maleic hydrazide to delay blossoming of fruits. — *Proc. Am. Soc. Hort. Sci.* 55: 147. 1950.
20. Wittwer, S. H. & Sharma, R. C.: Control of storage sprouting in onions by preharvest foliage sprays of maleic hydrazide. — *Science* 112: 597. 1950.
21. Zukel, V. W.: Maleic hydrazide as a plant growth inhibitor. — *Agric. Chemicals*, May, 1950.

Occurrence of Tyrosinase and Laccase in Fruit Bodies and Mycelia of some Hymenomycetes

By

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Considering the fact that the hymenomycetes play an important rôle in the breakdown of plant residues in nature, the question of their enzyme production is of great interest, and a good deal of work has been done in this field. With respect to the decomposition of lignin by the fungi a connection has been found between this activity and the ability of the mycelium to form enzymes of the phenol oxidase type (Bavendamm 1928, Davidson, Campbell and Blaisdell 1938, Lindeberg 1948 a).

The phenol oxidases which have so far been found in hymenomycetes seem to belong to one or the other of two enzyme types: tyrosinase and laccase.¹ These enzymes which are both copper proteins have much in common. However, they differ with respect to their specificity and their sensitivity to carbon monoxide, to elevated temperatures, &c. (for details and references see Dawson and Tarpley 1951). Both enzymes catalyze the oxidation of di- and polyphenols with two hydroxyl groups in the ortho position, e.g. catechol,

¹ The nomenclature of the phenol oxidases is rather confusing and is strongly in need of revision. At present the authors consider it preferable to use the two original names, tyrosinase and laccase. Since both these enzymes catalyze the oxidation of o-dihydric phenols, e.g. catechol, and polyphenols with two hydroxyl groups in the ortho position, e.g. pyrogallol and gallic acid, both possess a »catecholase» and »polyphenolase» activity. Therefore, it seems to us that the terms »catecholase» and »polyphenol oxidase» could scarcely be restricted to the one of the two enzymes. In accordance with this, one of the authors (L.) in earlier papers used the term »catecholase» for phenol oxidase, indicating an enzyme which oxidized catechol but which had not yet been defined by further investigations.

pyrogallol, and gallic acid. However, tyrosinase also catalyzes the oxidation of such monophenols as phenol and tyrosine, whereas laccase has no action on these compounds. p-Cresol is oxidized by tyrosinase to a coloured product of quinone structure whereas it is oxidized by laccase in another way, probably to a dicresol which forms a white precipitate (cf. Fåhræus 1949, p. 621). Laccase catalyzes the oxidation of hydroquinone and p-phenylenediamine which are not attacked by tyrosinase. The activity of tyrosinase is inhibited by carbon monoxide, this inhibition being independent of light. Laccase is insensitive to carbon monoxide. Tyrosinase is inactivated at a somewhat lower temperature than laccase; it is also precipitated by lower concentrations of ammonium sulphate and alcohol.

Studying the substrate range of the phenol oxidases which are secreted by certain wood-rotting fungi, Fåhræus (1949) found these enzymes to be essentially of the laccase type. However, in the cultivated mushroom, *Psalliota bispora*, Lindeberg (1950) found different parts of the fungus to form different phenol oxidases: the fruit bodies contain tyrosinase (or »polyphenol oxidase», Keilin and Mann 1938), the rhizomorphs tyrosinase and laccase, and the mycelium (cultivated in a nutrient solution) only laccase. Thus, the question arises whether tyrosinase and laccase have different physiological functions. Furthermore, one may ask if the distribution of these enzymes follows the pattern of *Psalliota bispora* in other lignin-decomposing species, and if, in non-lignin-decomposing species, e.g. the mycorrhiza-forming hymenomycetes, the distribution of the enzymes may possibly follow another pattern. Therefore, the present authors have studied the occurrence of phenol oxidases in fruit bodies, as well as mycelia grown on synthetic media, of some hymenomycetes which represent different ecological types, e.g. the litter-decomposing, the coprophilic, and the mycorrhiza-forming ones.

Materials and methods

The following species were studied:

Litter-decomposing species: *Clavaria ligula* Fr., *Clitocybe nebularis* Fr., *Lepiota procera* Fr., *Marasmius graminum* Berk. & Br. (only the mycelium), *M. peronatus* Fr., and *M. scorodoni* Fr. (cf. Lindeberg 1944, 1946).

Coprophilic species: *Psalliota arvensis* coll.

Mycorrhiza-forming species: *Boletus versipellis* Fr. (= *B. rufus*) and *Lactarius deliciosus* Fr. (cf. Melin 1923, 1925).

Fruit bodies of the fungi were collected in the autumn of 1950. Extracts of the fruit bodies were obtained in the following way: After addition of 1—2 parts of a 0.1 M phosphate buffer, pH 6.8, the fruit bodies were ground in a »Turmix» blender for one minute. After half an hour the suspension was centrifuged (3000

r.p.m.), and ammonium sulphate was successively added to the supernatant liquid to saturation. The mixture was then allowed to stand over night at $+2^{\circ}$ C. The fluid was removed by filtering through »celite» or by centrifuging. The precipitate was dissolved in a small amount of water and dialyzed against running tap water for about 12 hours. After filtering, the dialyzed solution was tested for its phenol oxidase activity.

Pure cultures of *Boletus versipellis* and *Lactarius deliciosus* were kindly placed at our disposal by Professor E. Melin, Uppsala. The other species were isolated by the authors either from spores or from the tissues of fruit bodies, »Hagem» malt-agar (Fries 1941, p. 274) being used as a substrate.

For the enzyme studies, the mycelia were cultivated floating on synthetic nutrient solutions in 125 ml Erlenmeyer flasks (Pyrex). Mostly 40 flasks, each containing 25 ml solution, were inoculated with one species. The cultures were incubated at 25° C for about 2—4 weeks. In some cases (*Bol. versipellis*, *Lact. deliciosus*) the incubation time was 6—8 weeks.

The following nutrient solutions were used:

A. Glucose 20 g, asparagine 1.5 g, KH_2PO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, Fe (as citrate), Zn, and Cu 1 p.p.m., Mn 3 p.p.m., Ca 20 p.p.m., aneurin 50 μg , yeast extract 100 mg, distilled water to 1000 ml. Used for the cultivation of *Clavaria ligula*, *Lepiota procera*, *Marasmius peronatus*, and *M. scorodonius*.

B. As solution A, but 4 g KH_2PO_4 and 1 g K_2HPO_4 instead of 1 g KH_2PO_4 . Used for *Clitocybe nebularis*.

C. As solution A, but 0.25 g KH_2PO_4 and 0.25 g K_2HPO_4 instead of 1 g KH_2PO_4 . Used for *Marasmius graminum*.

D. Glucose 10 g, asparagine 1 g, KH_2PO_4 0.5 g, K_2HPO_4 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, trace elements, aneurin, and yeast extract as in solution A, distilled water to 1000 ml. Used for *Psalliota arvensis*.

E. Glucose 10 g, NH_4 -tartrate 1 g, KH_2PO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, yeast extract 0.5 g, casein hydrolysate 0.25 g, tryptophan 5 mg, trace elements as in solution A, distilled water to 1000 ml. Used for *Boletus versipellis*.

F. Glucose 20 g, NH_4 -tartrate 0.5 g, KH_2PO_4 0.5 g, K_2HPO_4 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, yeast extract, casein hydrolysate, tryptophan, and trace elements as in solution E, distilled water to 1000 ml. Used for *Lactarius deliciosus* (cf. Melin & Norkrans 1948).

The mycelia were rinsed in distilled water. Extracts were obtained from the mycelia in the same manner as from the fruit bodies.

The phenol oxidase activity of the extracts, the specificity of the oxidases, and their sensitivity to carbon monoxide were studied by the Warburg technique. The following key was used for the characterization of the two enzyme types tyrosinase and laccase (cf. p. 101):

Enzyme	Oxidation of					Inhibition by CO
	catechol	hydroquinone	p-phenylenediamine	p-cresol	tyrosine	
Tyrosinase...	+	—	—	\pm^1	+	+
Laccase	+	+	+	\pm^2	—	—

¹ coloured oxidation product.

² no oxidation, or an oxidation followed by the formation of a white precipitate.

The following system was used in the Warburg experiments:

In the main compartment:

Enzyme-containing extract 1.0 or 2.0 ml.

Phosphate or citrate buffer 2.0 (0.1 *M*) or 1.0 (0.2 *M*) ml respectively.

In the side arm:

5 mg of the substrate dissolved in 0.3 ml distilled water. (In the case of tyrosine a suspension was used). When *p*-phenylenediamine is used as a substrate, the pH of the system shifts somewhat in the alkaline direction. This shift was compensated for by additions of the corresponding amounts of dilute HCl.

Total volume of fluid 3.3 ml.

The substrate was added at zero time. The experiments were run at 25° C. The respirometers were shaken at a rate of 85 complete strokes per minute.

The influence of CO on the enzyme activity was tested in experiments where the respirometers were filled with gas mixtures of 90 per cent CO + 10 per cent O₂ resp. 90 per cent N₂ + 10 per cent O₂ (control) by the evacuation method (Umbreit, Burris and Stauffer 1949, p. 44). The gases were obtained from tanks (CO from Matheson, U.S.A., N₂ and O₂ from AGA, Sweden). N₂ and CO were passed through alkaline pyrogallol before use.

Results

1. Phenol oxidases of fruit bodies and mycelia

The results of the investigations on the occurrence of phenol oxidases in the fruit bodies and mycelia of *Boletus versipellis*, *Lepiota procera*, and *Clavaria ligula* are to be found in Figs. 1—5. The extracts were tested at pH 5.6. With respect to their enzyme pattern these species represent three different types.

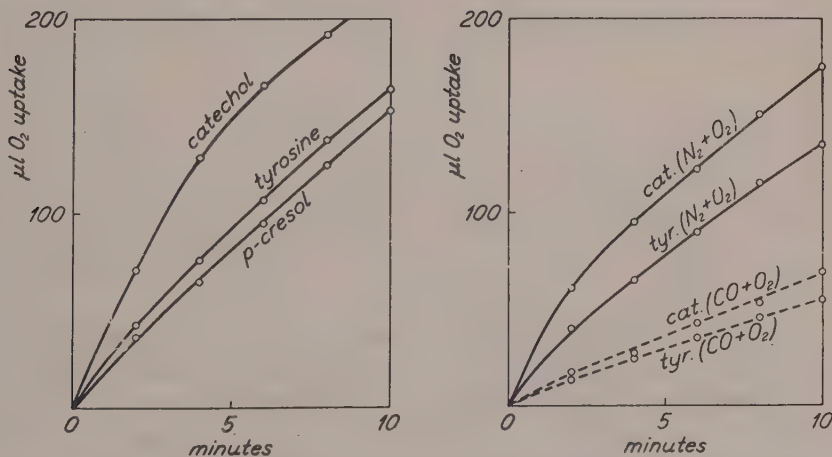


Fig. 1. Activity of an extract of fruit bodies of *Boletus versipellis*: A. Oxidation of various substrates. B. Effect of CO on the oxidation of catechol and tyrosine.

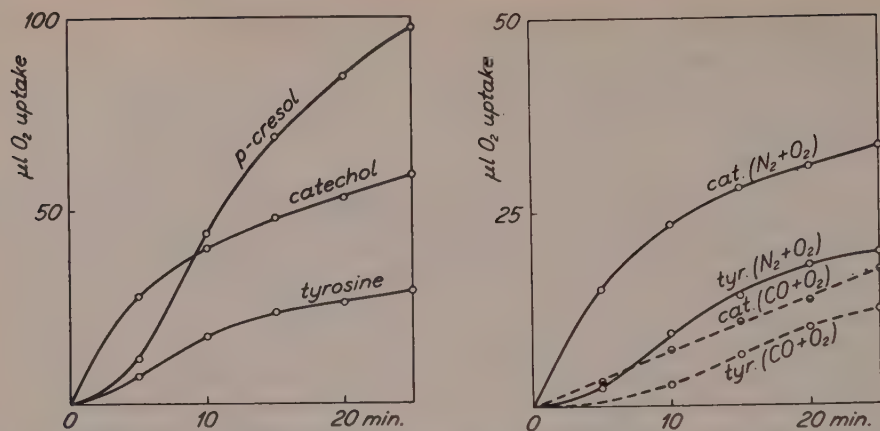


Fig. 2. Activity of an extract of mycelia of *Boletus versipellis*: A. Oxidation of various substrates. B. Effect of CO on the oxidation of catechol and tyrosine.

The extracts from fruit bodies and mycelia of *Boletus versipellis* catalyzed the oxidation of catechol, p-cresol (yellowish product), and tyrosine but not the oxidation of hydroquinone or p-phenylenediamine. The oxidation of catechol and tyrosine were both inhibited by carbon monoxide to about the same extent viz. 60—75 per cent. Thus, both extracts contained an enzyme of the tyrosinase type.

The fruit-body extract of *Lepiota procera* catalyzed the oxidation of hydroquinone and p-phenylenediamine as well as that of catechol, p-cresol, and

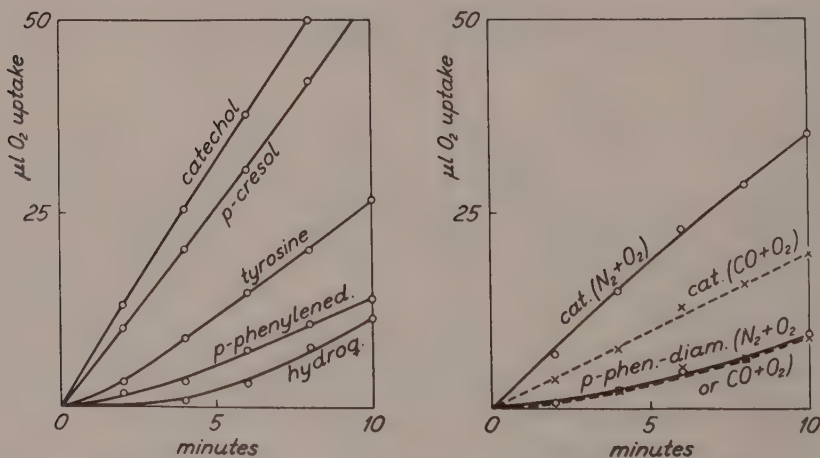


Fig. 3. Activity of an extract of fruit bodies of *Lepiota procera*: A. Oxidation of various substrates. B. Effect of CO on the oxidation of catechol and p-phenylenediamine.

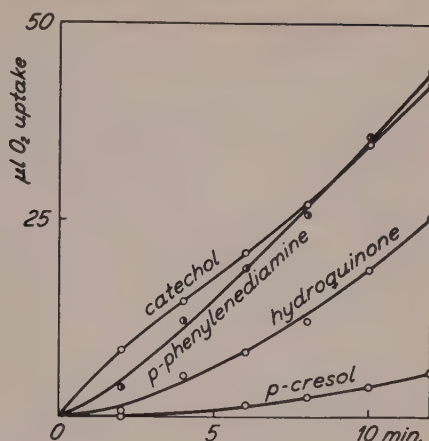


Fig. 4. Oxidation of various substrates by an extract of mycelia of *Lepiota procera*.

tyrosine. Carbon monoxide inhibited the oxidation of catechol, whereas the oxidation of p-phenylenediamine was not affected. However, the inhibition of the catechol oxidation in this case amounted to only about 45 per cent. These results show that the extract contained both tyrosinase and laccase.

The mycelial extract of the same species oxidized catechol, hydroquinone, and p-phenylenediamine. Tyrosine was not oxidized, whereas the oxidation of p-cresol was very weak, the oxidation product in this case having no colour. Neither the catechol oxidation nor the p-phenylenediamine oxidation was inhibited by carbon monoxide. Thus, the extract contained laccase but little or no tyrosinase.

The extract of fruit bodies of *Clavaria ligula* had a strong oxidizing effect on catechol, hydroquinone, and p-phenylenediamine, but had no action on p-cresol and tyrosine. Carbon monoxide had no effect on the oxidation. Thus, this species seems to contain only laccase.

Only small amounts of mycelium were obtained from this species, and the mycelial extract had only a weak activity. A weak oxidizing effect on catechol and hydroquinone could be observed whereas p-cresol and tyrosine were not attacked, indicating that the mycelium contained some laccase.

The results of the investigations on the remaining species are given in Table 1.

Both mycelial extracts and extracts from fruit bodies of *Clitocybe nebularis* contained mixtures of tyrosinase and laccase. However, the fruit-body extracts started the oxidation of tyrosine more rapidly than the mycelial extracts. The catechol oxidation was inhibited by carbon monoxide. In the fruit-body extract, this inhibition amounted to about 50 per cent, in the mycelial extract to about 30 per cent. The p-phenylenediamine oxidation was

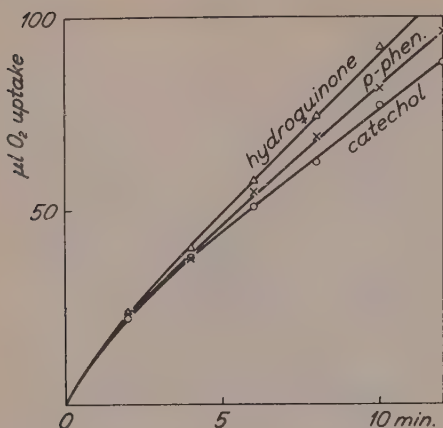


Fig. 5. Oxidation of various substrates by an extract of fruit bodies of *Clavaria ligula*.

not sensitive to carbon monoxide. The results seem to indicate that the fruit bodies contained relatively more tyrosinase than the mycelium.

From fruit bodies of *Marasmius peronatus*, extracts were obtained with typical tyrosinase activity. No phenol oxidase activity was shown by mycelial extracts of this species. However, when the fungus is cultivated on malt agar, containing gallic or tannic acid, dark-coloured zones are formed around the mycelium (Lindeberg 1944, 1948 a). Therefore, in one experiment the fungus was grown on »Hagem» malt agar to which in different series 0.05 per cent catechol, guaiacol, p-cresol, and tyrosine had been added. Coloured zones appeared around the mycelium on the catechol- and guaiacol-containing agar media but not on the media containing p-cresol or tyrosine. Thus, *M. peronatus* forms laccase on media, containing substances which are able to function as substrates for the enzyme, the enzyme in this case apparently being adaptive in nature.

Fruit-body extracts of *Marasmius scorodoni* were obtained only in small quantities, so that only a few experiments could be performed with each extract. The first extract oxidized catechol and this activity was inhibited by carbon monoxide to about 50 per cent. For the second extract an oxidizing activity towards hydroquinone and p-phenylenediamine could also be established. A weak oxidation of p-cresol and tyrosine was also observed. The mycelial extract of this fungus oxidized catechol and hydroquinone as well as p-cresol and tyrosine, the oxidation of catechol and tyrosine being inhibited by carbon monoxide to about 40–60 per cent resp. 80 per cent. Thus it is evident that the fruit bodies and mycelia of this species contained both tyrosinase and laccase.

Fruit bodies of *Marasmius graminum* were not obtainable. However,

Table 1. Enzyme activity of extracts from fruit bodies and mycelia of different hymenomycetes.

Species	pH of the system	Time in min.	$\mu\text{l O}_2$ uptake at the oxidation of					Inhibition by 90 % CO
			catechol	hydroquinone	p-phenylene-diamine	p-cresol	tyrosine	
<i>Clit. nebularis</i>								
fruit bodies	5.6	10	110	71	57	74	82	cat.: c. 50 %
mycelium	5.6	10	43	34	28	18	—	cat.: c. 30 %
»	5.6	30	—	—	—	—	23	—
<i>Mar. peronatus</i>								
fruit bodies	5.0	30	105	0	2	212	53	cat.: c. 50 %
mycelium	5.0	60	0	0	0	0	0	—
<i>Mar. scorodoni</i>								
fruit bodies (1)	5.6	10	56	—	—	—	—	cat.: c. 50 %
» » (2)	5.0	60	49	30	41	8	5	—
mycelium	5.0	20	135	39	—	60	23	cat.: c. 50 %
<i>Mar. graminum</i>								
mycelium	6.0	30	165	216	162	162	0	none
<i>Psall. arvensis</i>								
fruit bodies	6.8	10	179	146	260	7	2	none
mycelium	6.8	20	49	38	50	14	0	none
<i>Lact. deliciosus</i>								
fruit bodies	6.8	10	56	15	14	12	6	(none)
» »	6.8	30	—	—	—	88	54	—
mycelium	6.8	60	5	—	8	—	—	—

—: not tested.

according to earlier results (Lindeberg 1948 b) the mycelium of this species, when cultivated in synthetic media, forms a very active phenol oxidase. Therefore, mycelial extracts of this species were also studied and were found to possess typical laccase activity.

Extracts of fruit bodies and mycelia of *Psalliota arvensis*, which might possibly be referred to the coprophilic fungi from an ecological point of view, showed enzyme activities of the laccase type.

Finally, from fruit bodies of *Lactarius deliciosus*, an extract was obtained which oxidized catechol, hydroquinone, and p-phenylenediamine, as well as, after a certain induction period, p-cresol and tyrosine. Though no inhibition of the catechol oxidation could be observed in short-duration experiments (15 minutes), one must conclude that in addition to laccase the extract contained small amounts of tyrosinase.

Since the mycelium of *L. deliciosus* grows extremely slowly on synthetic nutrient solutions, only a small quantity was available for the preparation of an extract, the enzymic activity of which was very low. However, a weak

Table 2. Occurrence of tyrosinase and laccase in fruit bodies and mycelia of the species studied.

Species	Enzyme types	
	in fruit bodies	in mycelium
Litter-decomposing:		
<i>Clavaria ligula</i>	laccase	laccase
<i>Clitocybe nebularis</i>	tyrosinase + laccase	tyrosinase + laccase
<i>Lepiota procera</i>	tyrosinase + laccase	laccase
<i>Marasmius graminum</i>	—	laccase
» <i>peronatus</i>	tyrosinase	laccase (adapt.)
» <i>scorodonius</i>	tyrosinase + laccase	tyrosinase + laccase
Coprophilic:		
<i>Psalliota arvensis</i>	laccase	laccase
Mycorrhiza-forming		
<i>Boletus versipellis</i>	tyrosinase	tyrosinase
<i>Lactarius deliciosus</i>	(tyrosinase) + laccase	laccase

—: not tested.

oxidation of catechol and p-phenylenediamine could be established, indicating the occurrence of laccase.

The results of the investigations presented above have been summarized in Table 2.

2. Secretion of phenol oxidases into the medium

As mentioned above, a secretion of phenol oxidases into the medium is characteristic for litter-decomposing and white-rot fungi, when cultivated on media containing phenolic substances. However, a secretion of phenol oxidases into the medium by such fungi has been observed also when the fungi were grown on ordinary nutrient solutions without phenols or on sterilized litter (Lindeberg 1948 a, b, 1949). As pointed out by Lindeberg (l.c.), the extracellular phenol oxidases from fungi probably are of certain importance in the oxidation processes in the soil. Studying the enzyme activity of the mycelia of different species, the present authors found it worth while to investigate the secretion of phenol oxidases from the mycelia of certain species, these species representing different types with respect to the enzymes they contain.

The mycelia of the following species were chosen: *Boletus versipellis*, which contains only tyrosinase, *Marasmius scorodonius*, containing both tyrosinase and laccase, and *Marasmius graminum*, containing only laccase. At the end of the incubation period, the nutrient solutions were filtered, saturated with ammonium sulphate, and left over night at 2° C. The preci-

Table 3. *Enzyme activity of extracts of nutrient solutions from cultures of Marasmius scorodonius and Marasmius graminum.*

Species	pH of the system	Time in min.	μ l O ₂ uptake at the oxidation of					Inhibition by 90 % CO
			catechol	hydroquinone	p-phenylene-diamine	p-cresol	tyrosine	
<i>Mar. scorodonius</i>	5.0	30	103	73	85	16	0	none
<i>Mar. graminum</i>	6.0	30	160	217	174	182	0	none

pitates were then dissolved in small volumes of water and dialyzed. After filtering, the dialyzed solutions were tested for their enzyme activity (Table 3).

The medium in which *Boletus versipellis* had been grown showed no phenol oxidase activity.

The medium from cultures of *Marasmius scorodonius* contained a phenol oxidase which catalyzed the oxidation of catechol, hydroquinone, and p-phenylenediamine. Tyrosine was not attacked. The oxidation of catechol was insensitive to carbon monoxide. Thus, whereas the mycelium of *M. scorodonius* contained both tyrosinase and laccase, only the laccase was secreted into the medium.

Finally, the mycelium of *Marasmius graminum*, which contains a highly active laccase, secreted considerable amounts of this enzyme into the medium. The enzyme extracts from the mycelium and from the medium showed identic properties.

3. Influence of pH on the phenol oxidase activity

In order to further characterize some of the enzyme extracts described above, the influence of the hydrogen ion concentration on the activity was studied in certain cases, this influence being of special ecological interest. The results are shown in Fig. 6 A—D.

In the tyrosinase-containing extract from fruit bodies of *Marasmius peronatus*, a rather wide optimum zone was found between pH 3.5 and 5.5. After a certain amount of oxygen had been taken up, the enzyme was inactivated, as has been found with the tyrosinase from *Psalliota* (Ludwig and Nelson 1939).

With a mycelial extract of *Boletus versipellis*, which also contained tyrosinase, deviating results were obtained. At the pH values 5.4 and 6.9, this extract was inactivated, too, after a certain oxygen uptake. However, the extract was active also in the pH interval 3.8—2.8, but no inactivation

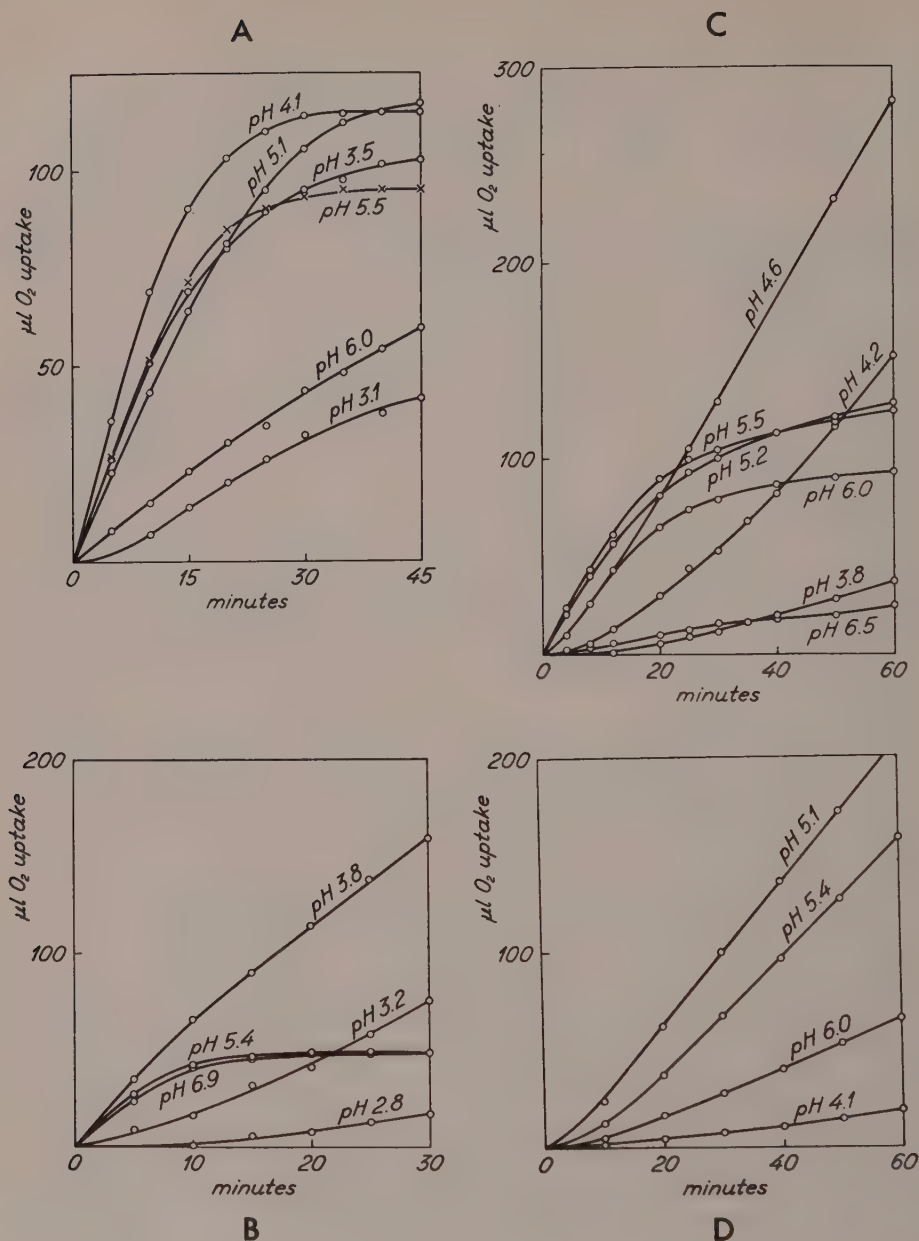


Fig. 6. Effect of pH on the oxidation of catechol by different extracts: A. Extract of fruit bodies of *Marasmius peronatus*. B. Extract of mycelia of *Boletus versipellis*. C. Extract of mycelia of *Marasmius scorodoni*. D. Extract of the nutrient solution from cultures of *Marasmius scorodoni*.

occurred at these pH values. If the extract had been heated to about 80° C for 15 minutes, no activity was obtained at pH 3.8. These results seem rather remarkable since the tyrosinase of *Psalliota* in systems buffered below pH 5 progressively shows lower activity, probably due to a dissociation of the copper from the enzyme (cf. Dawson and Tarpley 1951, p. 466).

The mycelial extract of *Marasmius scorodonius*, containing both tyrosinase and laccase, showed maximum activity at pH 5.2—5.5 during the first 15 minutes of the experiment. Later on, the enzyme activity in the pH interval 5.2—6.0 decreased on account of inactivation. At lower pH values, however, the extract behaved as that of *Boletus versipellis*, no inactivation occurring between pH 4.6 and 3.8. An extract of the medium in which *Marasmius scorodonius* had been cultivated and which contained laccase, had a maximum effect at about pH 5, whereas at pH 4.1 the activity was rather low. No inactivation could be observed during one hour, a result which coincides with the findings of Gregg & Miller (1940) on laccase from *Russula foetens*. According to these results it seems most probable that the activity of the mycelial extract at pH 4.6—4.2 was due both to tyrosinase and laccase.

The investigations on the activity of the mycelial extracts at low pH values are to be continued.

Discussion

It has been shown that in some species, e.g. *Lepiota procera* and *Marasmius peronatus*, as in *Psalliota bispora* (Lindeberg 1950), tyrosinase occurs only in the fruit bodies but not in the mycelium. On the other hand, the mycelium of *Marasmius peronatus* forms laccase as an adaptive enzyme, and in *Psalliota bispora* (l.c.) the mycelium, when cultivated in a synthetic medium, produces only laccase. Judging from these results there seems to exist in certain fungi a tendency to form tyrosinase in the fruit bodies and laccase in the mycelium. This tendency may perhaps be connected with the intra- and extracellular occurrence of the two enzymes.

According to the results presented above, tyrosinase occurs in the mycelia only as an intracellular enzyme. On the other hand laccase occurs both intra- and extracellularly. Although only a few species have been studied so far, there are reasons to assume that the two phenol oxidases do occur in the same way also in the mycelia of other hymenomycetes. Certain observations by other workers support this view. As mentioned above, Fåhraeus (1949) found that the mycelia of a number of white rot fungi secreted into the medium phenol oxidases of the laccase type, i.e. in these cases the laccase occurred as an extracellular enzyme. On the other hand, Law (1950), in-

vestigating extracts from mycelia of white and brown rot fungi, found certain brown rot fungi to contain phenol oxidases of the tyrosinase type only, whereas no enzymes of the laccase type were found in these species. Since brown rot fungi as a rule do not secrete phenol oxidases (negative Bavendamm's tests), the tyrosinase seems to occur only intracellularly also in these organisms.

Therefore, it appears most probable that in those cases where typical positive Bavendamm's tests are obtained, as with white rot fungi and coprophilic species, the extracellular enzymes involved are of the laccase type. In this connection, it is of interest that most of the fungi which have so far been found to form mycorrhiza with coniferous forest trees, e.g. species belonging to the genera *Amanita*, *Boletus*, *Clitopilus*, and *Rhizopogon*, give negative Bavendamm's tests (Lindeberg 1948 a), indicating the absence of laccase in these organisms. *Lactarius deliciosus* forms an exception from this rule. Finally, enzymes of the tyrosinase type seem to occur exclusively intracellularly both in certain white and brown rot fungi and in some mycorrhiza forming species.

Thus, of the two phenol oxidases, probably only laccase is of direct importance for the extracellular oxidation of phenolic compounds in the soil. Earlier investigations by Lindeberg (1948 b) on the influence of pH on the activity of extracellular phenol oxidases from fungi, as well as the results presented above concerning the activity of laccase from *Marasmius scorodonium*, indicate that under the pH-conditions prevailing in forest soils the effect of these enzymes is probably at its maximum.

Summary

1. The occurrence of phenol oxidases in fruit bodies and mycelia of various litter-decomposing, coprophilic, and mycorrhiza-forming hymenomycetes has been studied.

2. The oxidase activity of extracts which had been precipitated by ammonium sulphate and dialyzed was determined by manometric techniques. The specificity of the oxidases and their sensitivity towards carbon monoxide were studied.

3. The mycelia of the litter-decomposing and coprophilic species formed laccase. In addition, tyrosinase was found in the mycelia of certain litter-decomposing species. The fruit bodies of the fungi belonging to these groups contained either tyrosinase or laccase or both of these enzymes. A tendency to form tyrosinase in the fruit bodies and laccase in the mycelium was observed in certain species.

4. Two mycorrhiza-forming species were studied. *Boletus versipellis* contained tyrosinase, whereas *Lactarius deliciosus* contained laccase both in the fruit bodies and in the mycelium.

5. Laccase occurred both intra- and extracellularly, whereas tyrosinase was found only as an intracellular enzyme. There are reasons to assume that in those cases where typical positive Bavendamm's tests are obtained with hymenomycetes, the extracellular enzymes involved are of the laccase type.

6. The influence of pH on the activity of some of the extracts was studied. With tyrosinase from *Marasmius peronatus* an optimum zone was found within the pH region 3.5—5.5, whereas the optimum of laccase from *Marasmius scorodonius* was around pH 5.

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References

- Bavendamm, W.: Neue Untersuchungen über die Lebensbedingungen holzerstörender Pilze. II. Mitt.: Gerbstoffversuche. — Centralbl. Bakt. 2. Abt. 76: 172. 1928.
- Davíson, R. W., Campbell, W. A. & Blaisdell, D. J.: Differentiation of wood-decaying fungi by their reactions on gallic or tannic acid medium. — J. Agr. Res. 57: 683. 1938.
- Dawson, C. R. & Tarpley, W. B.: Copper oxidases. — Sumner & Myrbäck, The enzymes II, 1: 454. New York 1951.
- Fåhræus, G.: On the oxidation of phenolic compounds by wood-rotting fungi. — Ann. Agr. Coll. Sweden 16: 618. 1949.
- Fries, N.: Über die Sporenkeimung bei einigen Gasteromyceten und mykorrhizabildenden Hymenomyceten. — Arch. Mikrobiol. 12: 266. 1941.
- Gregg, D. C. & Miller, W. H.: A laccase from the wild mushroom, *Russula foetens*. — J. Am. Chem. Soc. 62: 1374. 1940.
- Keilin, D. & Mann, T.: Polyphenol oxidase. Purification, nature and properties. — Proc. Roy. Soc. London, Ser. B 125: 187. 1938.
- Law, K.: Phenol oxidases in some wood-rotting fungi. — Ann. Bot. 14: 69. 1950.
- Lindeberg, G.: Über die Physiologie ligninabbauender Bodenhymenomyzeten. — Symb. Bot. Ups. 8, Nr 2. 1944.
- On the decomposition of lignin and cellulose in litter caused by soil-inhabiting hymenomycetes. — Arkiv f. bot. 33 A, Nr 10. Uppsala 1946.
- On the occurrence of polyphenol oxidases in soil-inhabiting basidiomycetes. — Physiol. Plant. 1: 196. 1948 a.
- Some properties of the catecholases of litter-decomposing and parasitic hymenomycetes. — Physiol. Plant. 1: 401. 1948 b.
- Influence of enzymatically oxidized gallic acid on the growth of some hymenomycetes. — Sv. Bot. Tidskr. 43: 438. 1949.
- Phenol oxidases of the cultivated mushroom *Psalliota bispora* f. *albida*. — Nature 166: 739. 1950.
- Ludwig, B. J. & Nelson, J. M.: Inactivation of tyrosinase in the oxidation of catechol. — J. Am. Chem. Soc. 61: 2601. 1939.

- Melin, E.: Experimentelle Untersuchungen über die Birken- und Espenmykorrhizen und ihre Pilzsymbionten. — Sv. Bot. Tidskr. 17: 479. 1923.
- Untersuchungen über die Bedeutung der Baummykorrhiza. — Jena 1925.
- & Norkrans, B.: Amino acids and growth of *Lactarius deliciosus* (L.) Fr. — Physiol. Plant. 1: 176. 1948.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F.: Manometric techniques and tissue metabolism. 2nd ed. — Burgess Publishing Co. Minneapolis 1949.

The Significance of the Acid Growth-Inhibiting Substances for the Rest-Period of the Potato Tuber

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During the rest-period of the potato large quantities of growth-inhibiting substances occur in the potato peelings (Hemberg 1946 and 1947). As these substances disappear when the potato leaves the rest-period in a natural way (Hemberg 1946 and 1947) or when it is treated with rest-breaking media such as ethylenechlorhydrin (Hemberg 1949 a) or glutathione (Hemberg 1950), they have been assumed to be of significance for maintenance of the rest-period. Growth-inhibiting substances also appear to be of importance for the rest-period of tree buds (Hemberg 1949 b).

In the above-cited investigations on the rest-period of the potato only one variety, Magnum bonum, has been examined. In order to elucidate the rôle played by the growth-inhibiting substances in the case of the rest-period of the potato, it should be interesting, however, to study the behaviour of several potato varieties with rest-periods of different length.

Material and Methods

Material

For the present investigation the varieties Eigenheimer, Ackersegen, Magnum bonum and Birgitta have been chosen, their rest-period being on an average 5, 7.7, 13 and 15 weeks respectively, according to Emilsson (1949). The material was grown by the Institute for Plant Research and Cold Storage at Nynäshamn and was harvested on Sept. 16, 1949. After harvesting part

Table 1. *The degree of rest on different dates in four different varieties of potato stored at +5° C. The potatoes were harvested on Sept. 16, 1949. The determinations were made by Dr B. Emilsson of the Institute for Plant Research and Cold Storage at Nynäshamn according to the method given by Emilsson (1949, p. 200).*

Variety	Date of investigation	Degree of rest in %	Length of rest-period in weeks
Eigenheimer	3/10	20	4
	17/10	0	
Ackersegen	3/10	60	7
	8/11	0	
Magnum bonum	3/10	100	10
	14/11	10	
	28/11	0	
Birgitta	3/10	100	14
	14/11	70	
	12/12	70	
	28/12	0	

of it was stored there at +5° C and one day before each extraction samples were sent up to the Institute for Plant Physiology of the University of Stockholm for examination. Another part of the material has since Sept. 21 been stored at the Institute for Plant Physiology at a temperature of +20° C in a room into which fresh air was fanned during the whole storage period in order to prevent harmful effects from gas in the laboratory.

No determinations in respect of the length of the rest-period have been made by the author in connection with this investigation owing to the lack of suitable accommodation. Conclusions respecting the degree of the rest in the different varieties at the time of the experiments can, however, be drawn from the rest-period determinations made by Dr B. Emilsson at the Institute for Plant Research and Cold Storage with material derived from the same crop, see Table 1. These experiments concern potatoes stored at +5° C.

The buds of potatoes stored at +20° C generally began to sprout immediately after the end of the rest-period, whereas those of potatoes stored at +5° C did not begin to sprout until considerably later.

For the experiments concerning treatment of resting potatoes with ethylenechlorhydrin described at the end of this paper the Magnum bonum variety was used. This was grown at Thorsåker Farm in Uppland.

Extraction.

In the present investigation as in several earlier ones (Hemberg 1949 a and 1950) only the potato peelings have been studied. The potatoes were peeled

and the peelings extracted with peroxide-free ether during about 44 hours at $+5^{\circ}$ C. The ether was changed three times during this period.

Extracts were prepared each 14th day. A very large number of these extracts were, however, spoilt during the course of the work by the use of impure chloroform. The extracts are not preserved in ether but are transferred after extraction to chloroform (analytical reagent). A large number of the extracts were in this case dissolved in a chloroform containing impurities which entirely destroyed the auxins. In subsequent experiments chloroform purified in the laboratory was therefore used. Chloroform (analytical reagent) was used as original material; this had been freed from the alcohol added by shaking with water. After the water had been removed from the chloroform by having been kept over dried Na_2SO_4 for one night, the chloroform was twice shaken with concentrated sulphuric acid (analytical reagent). It was then freed from every trace of sulphuric acid by means of repeated shaking with distilled water. After the chloroform had then been kept for one night over dried Na_2SO_4 , it was distilled and to the distillation was added absolute alcohol in the proportion of 10 ml per 1000 ml chloroform. This purified chloroform does not destroy the auxin in the extracts and will keep for at least a year, in any case if placed in dark, well-filled bottles in a refrigerator.

Demonstration of the Inhibiting Substances

According to Hemberg (1949 a) potato peelings contain both neutral and acid inhibiting substances. In earlier investigations on the disappearance of the inhibiting substances in connection with the interruption of the rest-period (Hemberg 1946, 1947, 1949 a and 1950) no distinction has been made between these substances.

In the present investigation the total content of inhibiting substances has sometimes been determined on the one hand and, on the other, the neutral and the acid inhibiting substances have been studied separately. The acid and the neutral inhibiting substances are separated by fractionation according to Boysen Jensen's (1941) »method II».

When the content of growth-inhibiting substances is very large in relation to the content of growth-promoting substances, which is the case in impure extracts of potato peelings prepared in the autumn, one can get an idea of the amount of inhibiting substances by examining the ability of the extracts to inhibit the effect of indole acetic acid in the Avena test. The same method can also be used to demonstrate neutral inhibiting substances in the neutral fractions of extracts prepared in the autumn, for at that time the content of neutral auxin in the potato peelings is very small (Hemberg 1947).

In the acid fraction of extracts fractionated according to Boysen Jensen's (1941) »method II» there occur acid growth-inhibiting substances as well as acid growth-promoting substances. Consequently, the former cannot generally be demonstrated in the same way as the inhibiting substances in impure extracts or in the neutral fraction, as the fractionation has eliminated all the neutral inhibiting substances, with the result that the content of the remaining acid inhibiting substances is usually relatively small in relation to the content of acid auxin. This method can only be used if the content of acid auxin is particularly small in relation to the amount of acid inhibiting substances. One can form an idea, however, of the amount of acid inhibiting substances in extracts of the same plant material prepared at different times by drawing activity curves for these extracts (Hemberg 1951). This is done by transferring different amounts of the extracts to agar discs and testing by means of the *Avena* test. The results are plotted in a system of coordinates with the curvature of the test plants expressed in d on the ordinate and the amount of extract in the agar discs on the abscissa. Now if extracts prepared at different times contain the same amount of auxin but different amounts of acid inhibiting substances, the lower parts of the activity curves will coincide to a greater or lesser degree, while their upper parts will run differently. The curve of the extract richest in inhibiting substances bends off at lower d values than the curves of the extracts poorer in inhibiting substances.

The Occurrence of Growth-Inhibiting Substances and Acid Auxin at Different Times in the Peelings of Four Different Varieties of Potato

Content of Inhibiting Substances in Impure Extracts

In the present investigation the content of inhibiting substances in impure extracts was at first determined as described above. It was then found that growth-inhibiting substances occurred in impure extracts prepared from peelings of resting potatoes of all the four varieties. Extracts of *Magnum bonum*, however, contained slightly smaller amounts of inhibiting substances than extracts of the other three varieties. Extracts of the *Eigenheimer* and *Ackersegen* varieties prepared from not resting potatoes also contained, however, large quantities of inhibiting substances, even exceeding those in the extracts of resting potatoes of the same varieties. Impure extracts prepared from *Magnum bonum* and *Birgitta* that had left the rest-period were not examined.

If some special inhibiting substance is of importance for the rest-period it seems clear from these results that not all inhibiting substances are so. In any case the varieties Eigenheimer and Ackersegen must contain inhibiting substances that cannot be of importance for the rest-period, as these substances remain even after the rest-period has been interrupted.

When attempting to distinguish different groups of inhibiting substances with and without importance for the rest-period, it seems reasonable to examine neutral and acid inhibiting substances separately.

Neutral Growth-Inhibiting Substances

Of each of the four potato varieties three extracts have been studied. The first was prepared on Sept. 21, 1949, from potatoes stored at $+5^{\circ}$ C after harvesting on Sept. 16. The other two were prepared on Oct. 5 and Nov. 16 respectively from potatoes stored at $+20^{\circ}$ C since Sept. 21. According to Table 1, the potatoes stored at $+5^{\circ}$ C of all the four potato varieties were in a state of rest when the first extraction was made. On Oct. 5 20 per cent of the Eigenheimer were resting, 60 per cent of the Ackersegen and 100 per cent of the Magnum bonum and Birgitta. At the third extraction the Eigenheimer and Ackersegen had completely left the rest-period, while 10 per cent of the Magnum bonum and 70 per cent of the Birgitta were resting. According to Emilsson (1949, Tab. 11), however, the rest-period of potatoes

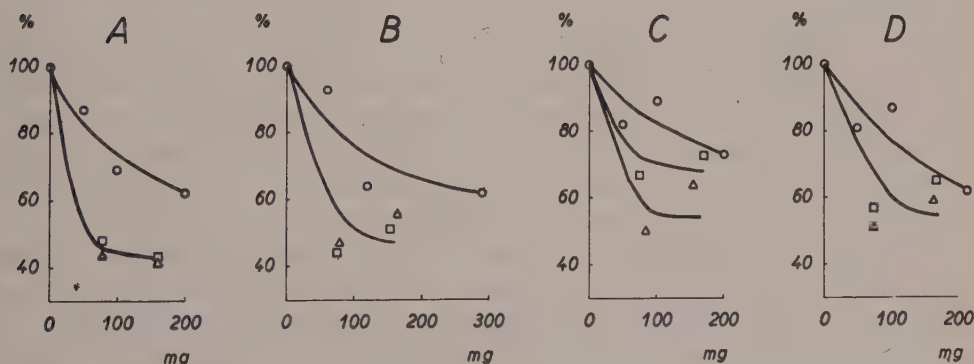


Fig. 1. The inhibition of the effect of indole acetic acid in the *Avena* test by neutral fractions of ether extracts prepared on different dates from peelings of four varieties of potatoes. Experiments with agar discs containing the same amount of indole acetic acid but varying amounts of extracts. A. Eigenheimer, B. Ackersegen, C. Magnum bonum, D. Birgitta. Extracts prepared on Sept. 21, 1949: \circ ; Oct. 5: \square and Nov. 16: \triangle respectively. Abscissa: mg of peelings corresponding to 0.1 ml agar. Ordinate: recovered amount of indole acetic acid in percentages of the amount in the control experiments.

stored at $+20^{\circ}\text{C}$ appears to be slightly shorter than that of potatoes stored at a lower temperature. It is therefore possible that the Eigenheimer had already completely left the rest-period at the time of the second extraction and the Magnum bonum at the time of the third.

The results (see Fig. 1) show that neutral inhibiting substances occur in all the extracts. Extracts from resting Eigenheimer, Ackersegen and Birgitta contain more inhibiting substances than those from resting Magnum bonum. The content of neutral inhibiting substances does not decrease during storage in any of the four varieties, but tends rather to increase. On the basis of these results it cannot be assumed that the neutral inhibiting substances are of any importance for the rest-period.

Acid Growth-Inhibiting Substances

Potatoes stored at $+20^{\circ}\text{C}$. — Eigenheimer. It will be seen from Fig. 2 A that extracts prepared on Sept. 21 from resting potatoes contain very little acid auxin. The amount of auxin is in fact so small and so little extract has been available that it has been impossible to draw an activity curve from whose course the occurrence of acid inhibiting substances, if present, could be established. Nor, owing to the low auxin content, can one form any idea of the content of inhibiting substance from the curve for the extract prepared on Oct. 5 from potatoes of which 20 per cent were resting. The third curve in Fig. 2 A represents two extracts prepared on Nov. 16 and Dec. 8 from not resting potatoes, whose auxin content had increased in connection with the sprouting.

Ackersegen. It will be seen from Fig. 2 B that the extract prepared on Sept. 21 contains much acid inhibiting substance, because the curve bends off and runs horizontally already at values of d about 0.30. The extract prepared on Oct. 5, when at most 60 per cent of the potatoes can have been resting, has a lower content of inhibiting substances, as shown by the fact that the curve for this extract tends to higher values of d than 0.30. The percentage of resting potatoes was probably lower than 60 per cent in that case, as the rest-period determinations, as already mentioned, apply to potatoes stored at $+5^{\circ}\text{C}$. The two curves are only separated at their uppermost point. Unfortunately, owing to the lack of extract, there is only one determination of the highest point for each curve. The variation between the separate determinations is, however, generally very small when the amounts of extract used are so large that the maximum values of d are reached for the extracts in question, whereas they can be larger when amounts of extract are used which give lower values of d . The difference between the two curves can therefore be regarded as significant.

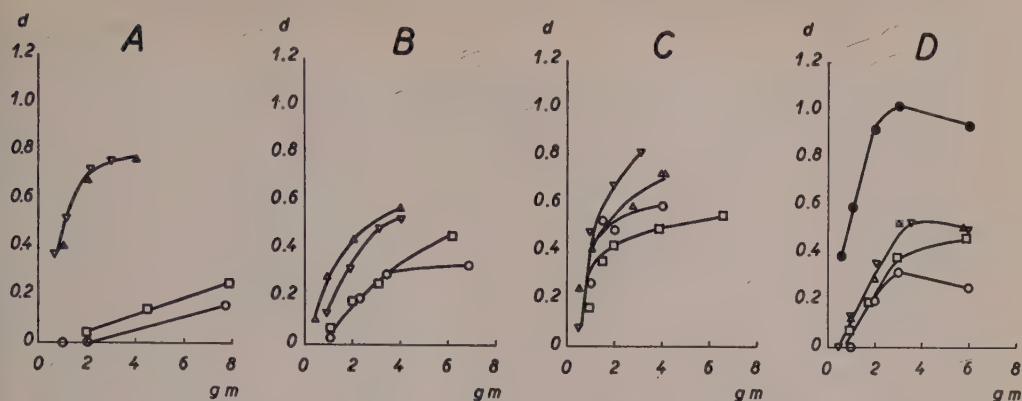


Fig. 2. Activity curves for the acid fraction of extracts prepared on different dates from peelings of four different varieties of potato stored at $+20^{\circ}$ C. A. Eigenheimer, B. Ackers-segen, C. Magnum bonum, D. Birgitta. Extracts prepared on Sept. 21: \circ ; Oct. 5: \square ; Nov. 16: \triangle ; Dec. 8: ∇ and Jan. 3: \bullet respectively. Abscissa: gram of peelings corresponding to 0.1 ml agar. Ordinate: curvature of test plants in d.

The extracts prepared at later dates contain slightly more auxin than the extracts prepared at earlier dates; consequently one cannot form any opinion of their content of inhibiting substances in relation to the extracts prepared at earlier dates.

Magnum bonum. It will be seen from Fig. 2 C that the content of inhibiting substances in extracts prepared from potatoes of which 100 per cent were resting is greater than in extracts from potatoes less completely at rest. The extract prepared on Oct. 5 contains besides more growth-inhibiting substances than that prepared on Sept. 21. Both of these extracts were prepared from potatoes in a 100 per cent state of rest. Similar observations have sometimes been made earlier too, i.e. that the content of inhibiting substances is greater in the middle of the rest-period than at its commencement.

When the potatoes have entirely left the rest-period the content of inhibiting substances further decreases, without an increase in the auxin content. The distribution of the values in the lower parts of the curves is not too great to prevent it from being regarded as falling within the limits of the experimental errors.

Birgitta. It will be seen from Fig. 2 D that the potatoes extracted on Sept. 21 contain the largest amount of inhibiting substance. The content diminishes in later extractions. On Dec. 8, when about 70 per cent were resting, the content of inhibiting substances was still comparatively large. The curves representing extracts prepared on Nov. 16 and Dec. 8 do not entirely coincide in their lower parts with the curves for extracts prepared

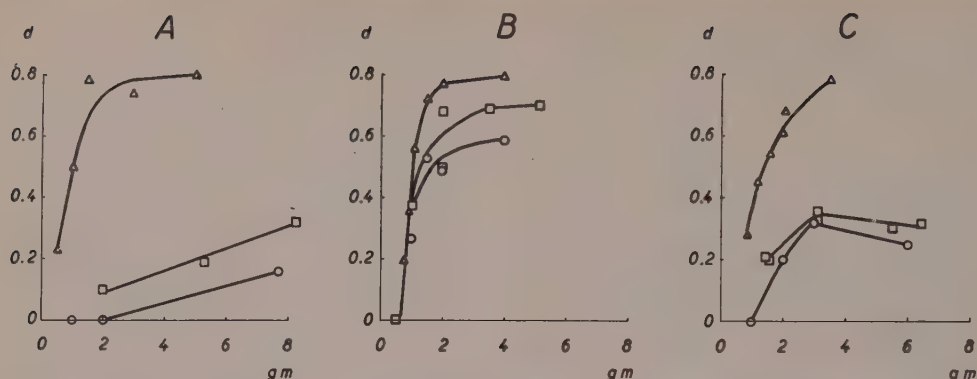


Fig. 3. Activity curves for the acid fraction of extracts prepared on different dates from peelings of three different varieties of potato stored at $+5^{\circ}\text{C}$. A. Eigenheimer, B. Magnum bonum, C. Birgitta. Extracts prepared on Sept. 21: \circ ; Dec. 7: \square and Jan. 26: \triangle respectively. Abscissa and ordinate as in Fig. 2.

earlier. The difference is so slight, however, that one may assume that all the four extracts contained about the same quantity of auxin.

In the extract prepared on Jan. 3 from potatoes that had completely left the rest-period the auxin content had increased considerably in connection with the sprouting; consequently nothing can be said about the content of inhibiting substances in relation to extracts prepared at earlier dates. Potatoes stored at $+20^{\circ}\text{C}$ can of course easily begin to sprout immediately the rest-period is completed, and in connection with this the auxin content increases.

Potatoes stored at $+5^{\circ}\text{C}$. — The Eigenheimer, Magnum bonum and Birgitta varieties have also been studied after being stored for varying periods of time at $+5^{\circ}\text{C}$.

Eigenheimer. The extract prepared on Sept. 21 from resting potatoes and that prepared on Dec. 7 from potatoes that had interrupted their rest-period contained so little auxin that it was impossible to form any opinion from the course of the activity curves of the content, if any, of acid inhibiting substances. The auxin content does not begin to increase until sprouting starts; see Fig. 3 A.

As the auxin content in the extracts prepared on Sept. 21 and Dec. 7 was so low, it was, however, possible to establish the acid inhibiting substances in the same manner as the neutral inhibiting substances, i.e. by studying the ability of the extracts to inhibit the effect of indole acetic acid in the Avena test. The results of these experiments (see Fig. 4) show that whereas the extract prepared on Sept. 21 from resting potatoes contains demonstrable amounts of acid inhibiting substances, the extract prepared on Dec. 7 from not resting potatoes contains practically nothing of these substances.

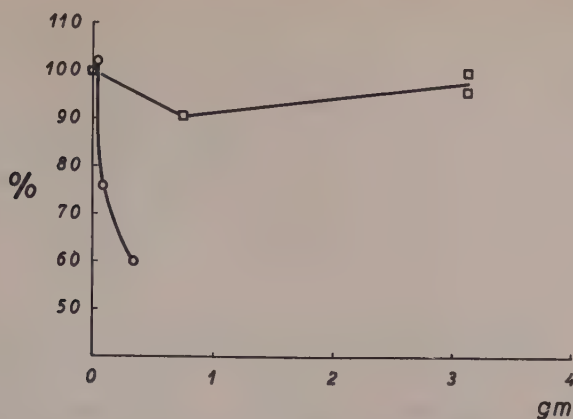


Fig. 4. The inhibition and non-inhibition of the effect of indole acetic acid in the *Avena* test by acid fractions of ether extracts from peelings of the *Eigenheimer* variety. Experiments with agar discs containing the same amount of indole acetic acid but varying amounts of extracts. Extracts prepared on Sept. 21: ○ and Dec. 7: □ respectively. Abscissa: gram of peelings corresponding to 0.1 ml agar. Ordinate: recovered amount of indole acetic acid in percentages of the amount in the control experiments.

Magnum bonum. The results of the experiments are shown in Fig. 3 B. Three extracts have been examined, prepared on Sept. 21, Dec. 7 and Jan. 26. The potatoes ceased resting at the end of November. It will be seen from Fig. 3 B that the content of acid inhibiting substances decreases in connection with the cessation of the rest-period.

Birgitta. The result (see Fig. 3 C) shows that much inhibiting substance is still present in the extract prepared on Dec. 7 from potatoes of which 70 per cent were resting, much more than in the extract prepared on Dec. 8 from potatoes stored at $+20^{\circ}$ C.

The potatoes stored at $+5^{\circ}$ C left the rest-period towards the end of December and those stored at $+20^{\circ}$ C probably earlier.

In the extract prepared on Jan. 26 from not resting potatoes the auxin increase occurring after the cessation of the rest-period and preceding the sprouting had begun, so that nothing can be said about this extract's content of inhibiting substances in relation to the other extracts.

Acid Auxin

The content of acid auxin in extracts prepared from resting potatoes of the four different potato varieties varies; see Table 2. It is lowest in the *Eigenheimer*, which has the shortest rest-period, and highest in the *Magnum bonum* with a comparatively long rest-period. The *Ackersegen* with a short

Table 2. *Amount of acid auxin in extracts of peelings of resting potatoes.* The potatoes were harvested on Sept. 16, 1949, peeled and extracted on Sept. 21, 1949, after storage at +5° C.

Variety	Amount of auxin expressed in WAE/1000 per gram of potato tissue
Eigenheimer	0.042
Ackersegen	0.148
Magnum bonum	0.369
Birgitta	0.171

rest-period and the Birgitta, which has the longest rest-period of the varieties studied, have roughly the same auxin content. Thus no connection can be proved between the auxin content during the rest-period and the duration of the rest-period. It will be seen from Figs. 2 and 3 that when the rest-period has ended the amount of auxin begins to increase. This occurs earlier in potatoes stored at +20° C than in those stored at +5° C; this is connected with the fact that sprouting takes place earlier in potatoes stored at the higher temperature. The increase in auxin seems to set in earlier in certain varieties, e.g. Birgitta, than in others, e.g. Magnum bonum.

The Effect of Ethylenechlorhydrin on the Acid Growth-Inhibiting Substances in the Potato Peelings

It has earlier been shown (Hemberg 1949 a) that the total content of inhibiting substances in the potato peelings decreases if the potatoes are treated with the rest-breaking medium ethylenechlorhydrin. In the preceding chapter it has been shown that while one cannot find any correlation between the occurrence of the neutral inhibiting substances and the rest-period, one can very well do so between the occurrence of the acid inhibiting substances and the rest-period. That being the case, the acid inhibiting substances should disappear from the potatoes when the resting potatoes are treated with ethylenechlorhydrin.

To test whether this assumption is correct, 6 kg of resting Magnum bonum potatoes were enclosed in a 28-litre container together with a wad of cotton-wool soaked in 12 ml of 40 per cent ethylenechlorhydrin. These potatoes are termed ethylenechlorhydrin-treated potatoes in the following. In another similar container were enclosed 6 kg of potatoes together with a wad of cotton-wool soaked in 12 ml of distilled water. These potatoes are termed not-treated potatoes in the following. Two days after the beginning of the treatment the potatoes were taken out and allowed to lie freely on a table for 24 hours, after which they were placed in new containers without ethylene-

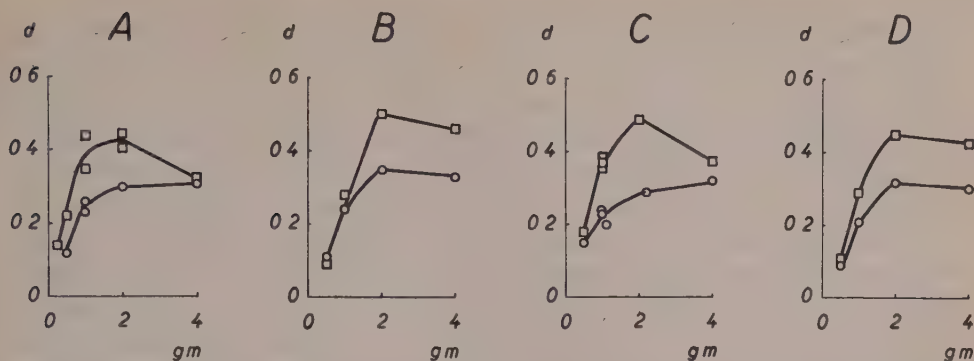


Fig. 5. Activity curves for the acid fraction of extracts of potato peelings prepared from ethylenechlorhydrin-treated potatoes and not-treated potatoes respectively on different dates after the beginning of the treatment. Not-treated potatoes: \circ ; ethylenechlorhydrin-treated potatoes: \square . A. 3 days; B. 5 days; C. 7 days; D. 9 days after the beginning of the treatment. Abscissa: gram of extract corresponding to 0.1 ml agar. Ordinate: curvature of the test plants in d.

chlorhydrin. The containers were kept at $+20^{\circ}$ C. 3, 5, 7 and 9 days after the beginning of the treatment samples of the ethylenechlorhydrin-treated potatoes as well as of the not-treated potatoes were taken out. The potatoes were peeled and the peelings extracted with ether. The extracts were purified according to Boysen Jensen's (1941) »method II» and the amount of acid inhibiting substance was established in the manner given above.

The results (see Fig. 5) show that the auxin content in the ethylenechlorhydrin-treated potatoes and in the not-treated potatoes is still the same 9 days after the beginning of the treatment. This is clear from the fact that the activity curves for extracts of ethylenechlorhydrin-treated potatoes and for not-treated potatoes coincide in their lower parts or at least in their lowermost point. The small deviations fall within the limits of the experimental errors. The activity curves also show, however, that the content of acid inhibiting substances in extracts from ethylenechlorhydrin-treated potatoes is considerably lower than in extracts from not-treated potatoes. Extracts from ethylenechlorhydrin-treated potatoes prepared three days after the beginning of the treatment already have a lower content of acid inhibiting substances than the corresponding extracts of not-treated potatoes.

Discussion

It has been shown in the foregoing that there are neutral as well as acid inhibiting substances in all the four varieties of potatoes studied. The neutral

inhibiting substances can occur in larger amounts in a potato variety with a short rest-period than in one with a longer rest-period. They do not disappear in connection with the cessation of the rest-period. Consequently, the neutral inhibiting substances cannot be of any significance for the rest-period. The acid inhibiting substances, on the other hand, which occur during the rest-period in all the varieties studied, disappear in connection with the potato leaving the rest-period. They disappear earlier from the Ackersegen with a relatively short rest-period than from the Magnum bonum and Birgitta varieties which have a longer rest-period. How soon they disappear from the Eigenheimer, which has the shortest rest-period, has not been determined, but they can be established in resting Eigenheimer whereas they have disappeared from the not resting Eigenheimer extracted on Dec. 7. It therefore seems probable that the disappearance also here is connected with the interruption of the rest-period.

In earlier experiments with potatoes (Hemberg 1946, 1947, 1949 a and 1950) only impure extracts were studied. In spite of this it has been possible to establish a disappearance of the inhibiting substances in connection with the cessation of the rest-period. This is due to the fact that the earlier experiments were made with the Magnum bonum variety, which, as shown in the foregoing, contains a smaller amount of neutral inhibiting substances than the other varieties studied. A disappearance of acid inhibiting substances can therefore be established in impure extracts of Magnum bonum even when the content of neutral inhibiting substances is unchanged.

Pohl (1951) has succeeded, by means of electrodialysis of extracts of potato peelings, in isolating a neutral inhibiting substance that occurs in larger amounts in young potatoes than in old ones. Pohl connects his discovery with Hemberg's investigations and believes that he has here isolated the neutral inhibiting substance previously described by Hemberg. But, as has been seen above, this does not disappear in connection with the cessation of the rest-period. Pohl's experiments, however, are difficult to compare straight away with those of Hemberg, as he has used *Lepidium* roots in his tests instead of *Avena coleoptiles* and has not given the exact date when the extracts were prepared. Neutral auxins that might occur may possibly be of some importance for the determinations.

Funke and Söding (1948) assume that the rest-period is regulated by a special hormone, antiauxin, with inhibiting effect. This is said to inhibit the growth of the buds during the rest-period, while the auxin stimulates it during the spring. These authors consider that the antiauxin can be changed into auxin. The antiauxin, however, is probably not identical with the acid inhibiting substances. These are not transferred into auxin at the end of the rest-period. The auxin content in the peelings of resting potatoes,

which are rich in acid inhibiting substance, and in the peelings of potatoes that have just left the rest-period and are poor in inhibiting substance, is, as we know, equally large.

It does not seem to be fully proved by Funke's and Söding's experiments that the antiauxin possesses any inhibiting effect. Pohl (1951) denies that the antiauxin is an inhibiting substance.

Meyer (1949) and Baumeister (1950) have also established inhibiting substances in potatoes, but they do not investigate the significance of these substances for the rest-period; instead they compare their presence in healthy and virus-infected potatoes.

Schulze and Fischnich (1951) as also Jähnl (1951) associate themselves with the present author's view that the rest-period in potatoes is regulated *inter alia* by growth-inhibiting substances.

In respect of earlier literature on the rest-period and inhibiting substances the reader is referred to the previous works of the present author.

The acid auxin is of no significance for the rest-period. This is clear from the fact that one cannot find any increase in the auxin content when the potatoes leave the rest-period and also from the fact that one cannot find any connection between the auxin content in one variety of potato and the length of the rest-period in that same variety.

Michener (1942) was of opinion that the rest-period in potatoes was due to the auxin content being supraoptimal during the rest-period. He said that when ethylenechlorhydrin treatment was employed the auxin content would decrease temporarily and the rest-period would then be interrupted. According to the experiments recorded in this paper the auxin content is at its lowest during the rest-period in all the varieties. The auxin content does not increase until the potatoes have left the rest-period and have begun to exhibit an ability to sprout. The small content of auxin during the rest-period cannot exert an inhibiting effect on the development of the potatoes; on the contrary this is stimulated by further additions of auxin in small doses, provided that the rest-period is simultaneously interrupted by rest-breaking substances (Hemberg 1949 a).

Summary

1. In the peelings of resting potatoes there are neutral as well as acid growth-inhibiting substances. In an investigation of four different varieties of potato with rest-periods of varying length it has been found that the acid inhibiting substances disappear when the potatoes leave the rest-period in a natural manner, but that the neutral inhibiting substances do not do so.

2. When resting potatoes are treated with the rest-breaking medium ethy-

lencechlorhydrin the acid inhibiting substances disappear from the peelings. This disappearance can already be observed 3 days after the beginning of the ethylenechlorhydrin treatment.

3. The acid growth-inhibiting substances are assumed to be of importance as regulators of the rest-period of the potato.

4. No connection exists between the auxin content during the rest-period and the length of that period. Thus the layer of peel of the Eigenheimer variety with a 4-weeks' rest-period contains least auxin and of the Magnum bonum variety with a 10-weeks' rest-period most auxin, whereas the auxin content in the same layer of the Ackersegen and Birgitta varieties with 7 and 14 weeks of rest comes in between these two extremes.

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References

- Baumeister, G.: Wuchs- und Hemmstoffe in der Knolle und im Kraut gesunder und abbaukranker Kartoffelpflanzen. — *Planta* 38: 683. 1951.
- Boysen Jensen, P.: Quantitative Bestimmung der beschleunigenden Streckungswuchsstoffe in der sauren Fraktion der Ätherextrakte aus höheren Pflanzen. — *Planta* 31: 653. 1941.
- Emilsson, B.: Studies on the rest period and dormant period in the potato tuber. — *Acta Agr. Suec.* 3: 189. 1949.
- Funke, H. & Söding, H.: Über das Wuchsstoff-Hemmstoffsystem der Haferkoleoptile und der Kartoffelknolle. — *Planta* 36: 341. 1948.
- Hemberg, T.: Wachstumshemmende und wachstumsfördernde Stoffe bei der Kartoffel. — *Arkiv f. Bot.* 33 B, No 2. 1946.
- Studies of auxins and growth-inhibiting substances in the potato tuber and their significance with regard to its rest-period. — *Acta Hort. Berg.* 14: 133. 1947.
- Significance of growth-inhibiting substances and auxins for the rest-period of the potato tuber. — *Physiol. Plant.* 2: 24. 1949 a.
- Growth-inhibiting substances in terminal buds of *Fraxinus*. — *Physiol. Plant.* 2: 37. 1949 b.
- The effect of glutathione on the growth-inhibiting substances in resting potato tubers. — *Physiol. Plant.* 3: 17. 1950.
- Establishment of acid growth-inhibiting substances in plant extracts containing auxins by means of the *Avena* test. — *Physiol. Plant.* 4: 437. 1951.
- Jähnl, G.: Die Wuchsstoffe in der Kartoffelknolle. — *Veröffentl. d. Bundesanst. f. alpine Landwirtsch. in Admont.* H. 4: 1. 1951.

- Meyer, E.: Über den Wuchsstoff-Hemmstoffgehalt gesunder und abbaukranker Kartoffelknollen. — *Planta* 38: 213. 1950.
- Pohl, R.: Das Hemmstoff-Wuchsstoffsystem des Maisskutellums. — *Planta* 39: 105. 1951.
- Schulze, W. & Fischnich, O.: Über Keimförderung und stoffliche Veränderungen in der Kartoffelknolle bei Beginn und im Verlauf der Keimung. — *Schriftenreihe der Forschungsanst. f. Landwirtsch. Braunschweig-Völkenrode H. 3: 5.* 1951.

Some New and Interesting Biochemical Mutations Obtained in *Ophiostoma* by Selective Enrichment Technique

By

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Introduction

The nutritional mutations so far isolated in microorganisms represent a rather large number of types, most of them requiring single amino acids, purines, pyrimidines, or vitamins. There is good reason to believe, however, that many more types actually exist, although they have escaped observation because of certain limitations in the isolation technique. This possibility had already been realized by the early Stanford group working with *Neurospora* (Beadle and Tatum 1945), and was later emphasized by various workers (Lein, Mitchell and Houlahan 1948, Pontecorvo 1950). Experience shows that there are three obvious possibilities of losing certain types of mutants using the ordinary methods of isolation:

- 1) the mutant may be extraordinarily rare;
- 2) the »complete» medium used for isolation may lack the particular factor (or factors) required by the mutant, or the ordinary cultural conditions as regards temperature, pH, etc. may be unsuitable for growth of the mutant;
- 3) the required factor is present in the isolation medium, but the mutant may be particularly sensitive to a metabolite analogue also present, which competes with the required factor, thus preventing the growth of the mutant.

These limitations of the ordinary isolation methods can, at least partially, be overcome by the following measures. By using an appropriate technique

the probability of obtaining even very rare types of mutations is increased. In preparing the complete medium, steps are avoided which may cause a destruction of chemically labile and heat-sensitive components of the medium. Certain ingredients, e.g. yeast extract and hydrolysed casein, which are known to contain inhibitory factors, are excluded or supplied in small amounts.

Employing these measures and using a carefully prepared liver extract as an extra supplement to the complete medium, we have performed a few experiments in order to search for mutants representing more unusual and, preferably, earlier unknown types in the Ascomycete *Ophiostoma multiannulatum*. In addition, since we were interested in all sorts of purine- and pyrimidine-deficient mutants, they were also included among those selected for.

Method

Conidia of *Ophiostoma* (strain No. 11, wild type) were produced in shaken tube cultures with a liquid nutrient solution composed of minimal medium (see below) plus 0.001 per cent inositol, 0.1 per cent asparagine, 2.5 per cent malt-extract, and 0.25 per cent casein hydrolysate. After filtration through cotton, the conidia were washed three times with distilled water by centrifugation. The suspension thus obtained was irradiated with UV until c. 99 per cent of the conidia had been killed.

Before plating, the majority of the unmutated living conidia was removed by means of the filtration procedure earlier described by Fries (1947). A somewhat modified type of filter was used, however. It consisted of a glass-filter tube for inverse filtration, 20×150 mm, with a porosity of 40–90 μ . A filter paper of appropriate size was placed between the filter plate and a supporting metal wire-net. The paper and the wire-net were then fixed by a piece of rubber tubing (with an inserted smaller rubber ring) put around the glass-filter tube.

In the first experiments the conidia were soaked in distilled water at +4° to +5° C for 72 hours after the UV irradiation. It appeared, however, that during this soaking the percentage of germinable conidia increased considerably, a fact which rendered the following treatment more difficult. This step in the procedure was therefore omitted, and immediately after irradiation the conidia were transferred to a liquid minimum solution at +25° C. After 12 to 24 hours, when the unmutated conidia had germinated, the suspension was filtered. 12 to 24 hours later this procedure was repeated once more with the filtrate, thus removing certain slowly-germinating wild type conidia. This second filtrate was appropriately diluted and plated out in petri-dishes with a complete agar medium.

The complete medium used in these experiments had the following composition: minimal medium (containing: glucose 20 g, ammoniumtartrate 5 g, KH_2PO_4 1 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, NaCl 0.1 g, CaCl_2 0.1 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 4.43 mg, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 4.05 mg, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 4 mg, thiamin 0.1 mg, pyridoxin 0.1 mg, and distilled water 1 liter) plus malt extract (Vitrum) 2 g, yeast extract (Difco) 0.2 g, hydrolysed casein (Casamino acids, Difco) 0.2 g, and agar 15 g. Furthermore, 0.5 ml liver extract, sterilized by Seitz-filtration, was added to each petri dish before the melted medium (at a temperature of c. $+40^\circ\text{C}$) was poured into the dish.

This liver extract was prepared in different ways, e.g. simply by extraction with distilled water (eventually followed by ultra-filtration and in some experiments concentration of the extract by evaporation *in vacuo*). However, the following procedure, which is essentially the method described by Dounce (1943), appeared to give the best results. 100 g fresh rabbit liver were ground for 15 to 20 minutes in a Turmix blender with 250 g ice and 250 g distilled water, to which was added 1.5 ml 1 M citric acid in order to adjust pH to c. 6. The extract was centrifuged for 1 hour at 10000—12000 r.p.m. (temperature $+4^\circ$ to $+5^\circ\text{C}$). If not needed immediately after preparation, the extract was stored in a frozen state.

In a few of the later experiments, the complete agar plates were incubated at $+19^\circ\text{C}$, whereas the rest of the procedure was performed as usual, at $+25^\circ$. In this way some temperature sensitive mutants could be obtained.

The monoconidial mycelia developing on this complete agar were transferred to »semi-complete» agar plates according to the ordinary method used in this laboratory (Fries 1948). The »semi-complete» medium contained, in addition to the constituents of the minimal nutrient solution, hydrolysed casein and a mixture of six vitamins (inositol, PABA, niacin, pantothenic acid, choline, and biotin). On this medium all wild type mycelia were able to grow, as well as mutant mycelia requiring amino acids and (most) vitamins. Those transfers which did not develop were consequently isolated and brought back to complete agar medium in tubes, being tentatively listed as mutants requiring nucleic acid constituents or unknown factors.

The nutritional requirements of the mutants were analysed by means of an auxanographic technique essentially similar to that employed by Pontecorvo (1947) and Perkins (1949).

Results

In view of the rather complicated series of consecutive steps comprising the whole procedure and the inherent variability of the organism, it was not surprising that many of the experiments failed, partially or completely. However, there were many sufficiently successful to provide us with a rich collection of mutants, most of which were deficient as to one or another nucleic acid constituent.

In the six experiments each of which had enabled the isolation of more than 400 monoconidial mycelia, the average percentage of mutants requiring constituents of nucleic acids amounted to 0.8 per cent, i.e. 41 mutants among 5058 mycelia tested. Since this category of mutants ordinarily represents c. 15 per cent of all biochemical mutations in *Ophiostoma* obtained by UV-irradiation, the percentage of all types counted together would amount to c. 5.4 per cent, which means an enrichment about five or six times.

Exclusive of the simple purine- and pyrimidine-less mutants, a number of types were obtained that had not earlier been found in *Ophiostoma*. Of the new mutations collected and at least preliminarily analysed, the following are the more notable:

W 1 requires asparagine, but growth with this single supplement is inferior to that of wild type. A further addition of methionine brings the growth up to the normal rate. This mutation seems to be different from that earlier found (No. 1518, Fries 1949), where asparagine could be exchanged for histidine, and no other substance was necessary for good growth.

W 3 grows normally only when supplied with adenine (or hypoxanthine), histidine, and glycine together. The single components have no effect, except a very weak one with adenine. After a lag period of about 6 days in shaken tubes, growth occurs with adenine+histidine, and with adenine+glycine after a somewhat shorter lag. Arginine exerts an inhibitory action. Glycine cannot be replaced by serine, and is active in a relatively high concentration. The mutation is probably the result of the change of a single gene, to judge from spontaneous back-mutations and the results of crossing experiments.

W 5 requires methionine+adenine, *W 134* uracil+biotin, *W 267* uracil+niacin (not tryptophan!), and *W 288* methionine+adenine (or hypoxanthine). All these strains are probably double mutants, although no definite evidence from crossing tests is available so far.

W 302 grows when supplied with glutathione. In the auxanographic tests, none of the three amino-acids constituting this tripeptide has any activity, neither alone, nor in combination with each other. Glutathione ($>0.3 \mu\text{mol}$ per 10 ml) exerts about the same activity when tested in the oxidized as in the reduced state. The dipeptide cysteinyl glycine, prepared by partial hydrolysis of glutathione (Kendall et al. 1930), has a growth effect as strong as that of the tripeptide.

W 66, *W 122*, *W 190*, *W 239*, *W 270*, *W 273*, *W 299*, and *W 306* are all temperature-sensitive mutants, which do not grow above a certain temperature limit, viz. $+25^{\circ}\text{C}$ or $+27^{\circ}\text{C}$. The last four mentioned above behave as wild type strains below this critical temperature. The others do not grow even at the lower temperature unless supplied with some essential compound, viz. inositol in *W 66* and *W 190*, lysine in *W 122*, and a vitamin+an

amino-acid (both unidentified as yet) in *W 239*. These four strains are obviously double mutants, and the coupling of temperature-sensitivity with a nutritional deficiency seems therefore only to be incidental. Although various media were tested, growth above $+25^{\circ}\text{C}$ could not be obtained with any of the strains, except *W 299* and *W 306*, where the critical temperature was $+27^{\circ}\text{C}$.

Summary

Nutritional mutations of *Ophiostoma multiannulatum* representing biochemical types not hitherto isolated were selected for by means of an enrichment technique. The complete medium used contained a carefully-treated liver extract. Among the more notable single-gene mutations obtained, one required the tripeptide glutathione, another one a combination of adenine, histidine, and glycine. Some of the other mutants with complex requirements were probably double mutations. A number of temperature-sensitive mutants were also isolated, none of them being capable of growth above $+25^{\circ}\text{C}$, or — in two cases — above $+27^{\circ}\text{C}$.

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References

- Beadle, G. W. & Tatum, E. L.: *Neurospora* II. Methods of producing and detecting mutations concerned with nutritional requirements. — *Amer. J. Bot.* 32: 678. 1945.
- Dounce, A. N.: Enzyme studies on isolated cell nuclei of rat liver. — *J. Biol. Chem.* 147: 685. 1943.
- Fries, N.: Experiments with different methods of isolating physiological mutations of filamentous fungi. — *Nature* 159: 199. 1947.
- Viability and resistance of spontaneous mutations in *Ophiostoma* representing different degrees of heterotrophy. — *Physiol. Plant.* 1: 330. 1948.
- X-ray induced physiological mutations in *Ophiostoma*. — *Proc. Fourth Intern. Congr. Microbiol.* 1947: 385. Copenhagen 1949.
- Kendall, E. C., Mason, H. L., & McKenzie, B. F.: A study in glutathione. IV. Determination of the structure of glutathione. — *J. Biol. Chem.* 88: 409. 1930.
- Lein, J., Mitchell, H. K. & Houlahan, M. B.: A method for selection of biochemical mutants of *Neurospora*. — *Proc. Nat. Acad. Sci. Washington* 34: 435. 1948.
- Perkins, D. D.: Biochemical mutants in the smut fungus *Ustilago maydis*. — *Genetics* 34: 607. 1949.
- Pontecorvo, G.: The genetical control of nutritional requirements in micro-organisms and its application to microbiological assays. — *Proc. Nutrition Society* 5: 182. 1947.
- New fields in the biochemical genetics of micro-organisms. — *Biochem. Soc. Symposia* 4: 40. 1950.

Unterschiede der Plasmapermeabilität in den Gewebeschichten krautiger Stengel

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Permeabilitätsunterschiede zwischen verschiedenen Geweben derselben Pflanze sind heute schon in grösserer Zahl bekannt. Die ersten Beobachtungen stammen von Höfler und Stiegler (1921, 1930). Sie zeigten, dass die Stengel-epidermis von *Gentiana Sturmi* für Harnstoff vielmal permeabler ist als die darunterliegende subepidermale Parenchymschicht. Später fand Höfler (1936, 1937), dass bei *Gentiana Sturmi* auch die Korollröhre ein von den Stengelepidermiszellen völlig abweichendes Permeabilitätsverhalten zeigt. Bei ersterer permeiert Glyzerin, bei letzteren Harnstoff rascher durchs Plasma. Hurch (1933) konnte an Blättern verschiedener Pflanzen für mehrere Diosmotika gleichfalls eine durchweg schnellere Permeation in den Epidermis- als in den Subepidermiszellen feststellen. Nach den Angaben der Deplasmolysezeiten in der Untersuchung von Schmidt (1939) permeieren Harnstoff, Glyzerin und Malonamid in die Stengelepidermiszellen auch von *Lamium maculatum* schneller als in die Zellen der Subepidermis. In der Folge sind diese für die protoplasmatische Anatomie und vergleichende Protoplasmatik interessanten Ergebnisse verhältnismässig wenig beachtet worden.

Erst in neuerer Zeit wandte sich im Zusammenhang mit Untersuchungen über Verschiedenheiten der Gewebeschichten krautiger Blütenpflanzen (Höfler 1951, Kasy 1951) das Interesse auch wieder dem unterschiedlichen Permeabilitätsverhalten von Epidermis und Subepidermis zu. Höfler (1951) erinnert an die Entdeckung Hans Winklers, wonach bei den Periklinalchimären schon am Vegetationskegel verschiedene kappenartig übereinanderliegende Zellschichten zu beobachten sind, die aus Initialzellen hervorgehen,

die teils von dem einen, teils von dem anderen Pfropfpartner herrühren. Unterscheiden sich diese — wie bei *Solanum lycopersicum* und *Solanum nigrum* — in ihrer Chromosomenzahl, so ist darin ein vortreffliches Hilfsmittel gegeben, die aus diesen verschiedenen Meristemschichten hervorgehenden Gewebspartien an der fertigen Pflanze wiederzuerkennen. (Lange 1927). Nun erfahren aber auch in der normalen, genetisch einheitlichen Pflanze die Zellen im Urmeristem des Vegetationskegels durch inäquale Zellteilungen eine frühzeitige Determination, die sich gleichfalls in der Gewebeschichtung der fertigen Pflanze widerspiegeln muss.

Höfler regte nun an, auch da, wo die Unterscheidungsmöglichkeit nach der Chromosomenzahl wegfällt, nach anderen, zellphysiologisch greifbaren unterscheidenden Merkmalen der einzelnen Gewebeschichten zu suchen. Die oben angeführten Permeabilitätsunterschiede zwischen Epidermis und Subepidermis gaben für diese Fragestellung eine erste Beobachtungsgrundlage.

Auch meine plasmometrischen Messungen an Zellen der Stengel verschiedener krautiger Pflanzen (Url 1951) zeigten wieder, dass die Permeabilität der Epidermis für verschiedene Anelektrolyte (Harnstoff, Glycerin, Methylharnstoff, Erythrit) allgemein grösser ist als die Durchlässigkeit der subepidermalen Parenchymschichte, darüber hinaus aber, dass diese Unterschiede zumeist eine gewisse Grössenordnung nicht übersteigen. So geht die Plasmolyse im überwiegenden Teil der Fälle in der Epidermis nicht mehr als 3 bis 4 mal schneller zurück als in der Subepidermis. Nur der Harnstoff macht zuweilen eine Ausnahme. In Fällen wo die Epidermis dem »rapiden Harnstofftyp« (*Gentiana-Sturmiana-Typ*) angehört, und wo der Harnstoff wesentlich schneller permeiert als es seiner relativen Lipoidlöslichkeit entspricht,¹ schneller als der besser lipoidlösliche aber grössermolekulare Methylharnstoff, fand ich an einigen Objekten, dass der Quotient der Rückdehnungsgeschwindigkeit von Epidermis und Subepidermis Werte bis über 10 erreicht.

1. Versuche mit Homogyne alpina

Kasy (1952) untersuchte zur Frage der protoplasmatischen Verschiedenheit der einzelnen Gewebeschichten stoffliche Unterschiede der *Zellsäfte*, deren Chemismus ja auch als ein Ausdruck der unterschiedlichen Tätigkeit der lebenden Protoplasten anzusehen ist. Besondere Aufmerksamkeit widmet

¹ Von »porengeförderter« Harnstoffpermeabilität sprechen die meisten Autoren dort, wo Harnstoff ebenso schnell oder schneller als Methylharnstoff permeiert. In den meisten Fällen geht hingegen wie bekannt der besser lipoidlösliche Methylharnstoff schneller durchs Plasma, und zwar meist 2—6 mal schneller. (Vgl. Collander und Wikström 1949).

die Autorin der Verteilung des Anthokyans im Stengel der höheren Pflanze. Bei verschiedenen Pflanzenfamilien zeigen sich häufig Beschränkungen des Anthokyan gehaltes auf bestimmte Gewebeschichten. So führen Cruciferen oder Papilionaceen fast ausnahmslos in der Subepidermis Anthokyan, bei Labiaten und Gentianaceen ist meist die Epidermis allein gefärbt. Es wäre nun zu denken, dass in diesen Gewebeschichten gleichlaufend mit der verschiedenen Fähigkeit zur Anthokyanbildung auch bestimmte Permeabilitätseigenschaften der Protoplasten einhergehen. Wie die folgenden Ausführungen zeigen, ist das aber nicht der Fall.

Ein für vergleichende Untersuchungen der Permeabilitätseigenschaften günstiges und interessantes Objekt fand sich im Blütenstiel von *Homogyne alpina*. Epidermis und Subepidermis sind hier beide rot; die Zellsäfte sind durch Anthokyan fast gleichstark gefärbt. Die Zellen der Subepidermis unterscheiden sich von denen der Epidermis nur dadurch, dass sie einige grosse grüne Plastiden besitzen. Neben den regelmässigen, für plasmometrisches Arbeiten gut geeigneten Zellformen ist die meist fast gleiche Grösse der epidermalen und subepidermalen Zellen ein weiterer Vorteil des Objektes, da ein direkter Vergleich der Permeabilitätswerte ohne eine Umrechnung auf die Oberflächeneinheit des Protoplasten erfolgen kann.

Die im folgenden beschriebenen Versuche wurden nach der *plasmometrischen Methode* von Höfler ausgeführt, von der genaue Darstellungen vielfach vorliegen (Höfler 1918, 1934 b, Hofmeister 1935, Elo 1939, Kreuz 1941, Biebl 1948). G, der Grad der Plasmolyse errechnet sich aus der plasmometrischen Grundgleichung:

$$G = \frac{1 - \frac{b}{h}}{\frac{b}{h}}$$

l stellt die Länge des Protoplasten (nach Abrundung) dar, h die innere Zelllänge und b die innere Zellbreite. Wir begnügen uns im folgenden, als Mass der Permeabilität ΔG zu verwenden, die Änderung des Plasmolysegrades in der Stunde.¹ ΔG errechnet sich aus $G_2 - G_1$ also der Differenz der Plasmolysegrade, welche auf die Stunde bezogen wird.

Folgende Versuchsprotokolle zeigen die deutlichen Permeabilitätsunterschiede zwischen Epidermis und Subepidermis des Stengels von *Homogyne alpina*.

Bei einem Versuch vom 5. Juli 1951 wurde ein Schnitt um 16^h41'30" in eine 1.2 molare Glyzerinlösung eingelegt. Von fünf gemessenen Zellen der Epidermis waren um 17^h32' eine deplasmolysiert, zwei zeigten Grenzplasmolyse und die beiden anderen Plasmolysegrade von 0.856 und 0.794. Erst

¹ Zwischen der Permeationskonstanten des Protoplasten P' (vgl. Collander und Bärklund 1933) und der Rückdehnungsgeschwindigkeit ΔG besteht die Beziehung $P' = \Delta G \frac{C}{C - c}$ wo C = die Aussenkonzentration des Diosmotikums und c = die Partialkonzentration desselben im Zellsaft.

Tabelle 1.

1.3 mol Glycerin

t = 26° C.

7. Juli 1950.

In die Lösung eingelegt um 11h13'.

Epidermis: 1. Messung 11h38', 2. Messung 11h58', 3. Messung 12h08', 4. Messung 12h18'.

Zelle	h	b	1 ₁	1 ₂	1 ₃	1 ₄	G ₁	G ₂	G ₃	G ₄	ΔG ₁₋₂	ΔG ₂₋₃	ΔG ₃₋₄
1	73	9	32	42	46	51	0.397	0.534	0.589	0.657	0.411	0.330	0.408
2	62	10	31.3	41	45	49.7	0.450	0.607	0.671	0.746	0.471	0.384	0.450
3	60	10	31	42	46	50.3	0.461	0.644	0.710	0.782	0.549	0.396	0.432
4	67	10	35.9	49.2	54.9	Grenz- plasmo- lyse	0.485	0.684	0.770	—	0.557	0.516	—
5	58	9.5	29.8	38	41	45	0.459	0.601	0.652	0.720	0.426	0.306	0.408
6	64	9	34.8	47	51	56	0.496	0.687	0.750	0.829	0.573	0.378	0.426

ΔG(Mittel) = 0.436

Subepidermis: 1. Messung 11h43', 2. Messung 12h03', 3. Messung 12h23', 4. Messung 12h53', 5. Messung 14h23'.

Zelle	h	b	1 ₁	1 ₂	1 ₃	1 ₄	1 ₅	G ₁	G ₂	G ₃	G ₄	G ₅	ΔG ₁₋₂	ΔG ₂₋₃	ΔG ₃₋₄	ΔG ₄₋₅
1	100	13.5	38	40	42	47	57	0.335	0.355	0.375	0.425	0.525	0.060	0.060	0.100	0.200
2	90	14	38	40	42	48.3	63	0.370	0.392	0.415	0.485	0.648	0.066	0.069	0.140	0.326
3	95	15	37	38.1	40	43.9	52	0.336	0.348	0.368	0.410	0.495	0.036	0.060	0.084	0.170
4	125	15	46	47.5	49	60	67	0.328	0.335	0.352	0.441	0.496	0.021	0.021	0.178	0.110
5	98	13	39	44	50	62	—	0.354	0.405	0.466	0.588	—	0.153	0.183	0.244	—

ΔG(Mittel) = 0.120

17h52' (also 20 Minuten später) erfolgte die erste Messung an der Subepidermis. Der durchschnittliche Plasmolysegrad der hier gemessenen (ebenefalls fünf) Zellen betrug 0.555.

Ein anderer Glycerinversuch zeigt den Unterschied ebenso deutlich (Tabelle 1).

Bei diesem Versuch erfolgte die letzte Messung der Epidermiszellen etwa im Zeitpunkt der dritten Messung der Subepidermis. Ein Vergleich der zugehörigen mittleren Plasmolysegrad-Werte ergibt für die Epidermis 0.747, für die Subepidermis aber 0.395! Die ansteigenden ΔG-Werte für die Subepidermis sind nicht etwa auf eine pathologische Erhöhung der Permeabilität zurückzuführen, sondern darauf, dass in diesem Fall wegen der zeitlichen Vergleichsmöglichkeit mit der Epidermis die Subepidermis-Messungen so früh angesetzt sind, dass sie erst den Beginn des Permeationsvorganges erfassen. Der durchschnittliche ΔG-Wert für die Subepidermis ist deshalb auch zweifellos niedriger als der tatsächliche. Aus diesem Grund ist es praktisch auch fast immer erforderlich — und es gilt dies auch für *Homogyne alpina* — bei vergleichenden Permeabilitätsmessungen die Messung der Subepidermis viel später, meist erst nach Beendigung der Epidermis-Messungen, vorzunehmen.

Das ist natürlich ganz besonders bei solchen Objekten der Fall, wo die Epidermis

Tabelle 2.

 1.2 mol Harnstoff $t=23^{\circ}\text{C}$. 5. Juli 1951.

Eingelegt in die Lösung 15h51'.

Epidermis: 1. Messung 16h, 2. Messung 16h03', 3. Messung 16h07', 4. Messung 16h10'.

Zelle	h	b	1 ₁	1 ₂	1 ₃	1 ₄	G ₁	G ₂	G ₃	G ₄	ΔG_{1-2}	ΔG_{2-3}	ΔG_{3-4}
1	66	5	52	55	60.2	64	0.762	0.807	0.888	0.944	0.900	1.215	1.120
2	49	5	38.5	41	44	46.5	0.752	0.803	0.864	0.915	1.020	0.915	1.020
3	35	5	29.8	31.2	33.3	35	0.804	0.844	0.905	0.952	0.800	0.915	0.940
4	37	6	31	32.6	34.6	Grenz- plasmolyse	0.784	0.826	0.881	—	0.840	0.825	—

 $\Delta G(\text{Mittel}) = 0.955$

16h13' waren Zelle 2 und 4 deplasmolysiert, Zelle 1 und 3 zeigen eine schwache Grenzplasmolyse.

Subepidermis: 1. Messung 16h18', 2. Messung 16h30', 3. Messung 16h36', 4. Messung 17h16'.

Zelle	h	b	1 ₁	1 ₂	1 ₃	1 ₄	G ₁	G ₂	G ₃	G ₄	ΔG_{1-2}	ΔG_{2-3}	ΔG_{3-4}
1	47	6	—	36.8	37.8	43	—	0.740	0.762	0.872	—	0.220	0.165
2	61	6	44.1	46	47.2	55.2	0.690	0.721	0.741	0.872	0.155	0.200	0.196
3	81	6	63.5	65.7	67.2	77	0.759	0.782	0.805	0.926	0.115	0.230	0.181
4	55	6	44	46	47.3	Grenz- plasmolyse	0.764	0.800	0.824	—	0.180	0.240	—
5	52	6.5	40	41.2	42	48.5	0.727	0.751	0.766	0.890	0.120	0.150	0.186

 $\Delta G(\text{Mittel}) = 0.180$

einem »rapiden Harnstofftyp« entspricht. Um in der Epidermis ausreichende Plasmolysen zu erzielen, muss man hier mit hohen Konzentrationen von 2.0 oder 1.8 mol Harnstoff arbeiten. Dabei ist es die Regel, dass zum Zeitpunkt der Deplasmolyse der Epidermis die Subepidermis noch keineswegs gerundete Protoplaste, sondern noch stark konkave Plasmolysen aufweist. Zuweilen ist es deshalb auch notwendig, die Messungen an der Subepidermis gesondert in geringeren Konzentrationen vorzunehmen (vgl. Url 1951, S. 486). Das war aber bei Homogyne alpina nicht nötig.

Tabelle 2 gibt den Verlauf eines Harnstoffversuches an Homogyne alpina wieder.

Bei diesem Versuchspaar setzt die Messung der Subepidermis erst nach Beendigung der Messungen an der Epidermis ein. Trotzdem sind die G-Werte der ersten Epidermis-Messung schon deutlich höher als die 18 Minuten später gemessenen G-Werte der ersten Subepidermis-Messung. Die plasmometrisch gewonnenen ΔG -Werte sind durch die beobachteten Deplasmolysezeiten gestützt. Diese betragen für die Epidermis 24 Minuten, für die Subepidermis

110—112 Minuten. Daraus ergeben sich — nach Hofmeister (1948) — folgende ΔG_d -Werte: Epidermis: 1.100, Subepidermis: 0.250.

Die Berechnung von ΔG_d (ΔG gewonnen aus einem Deplasmolysezeitversuch) erfolgt bei der Permeabilitätsbestimmung nach der Deplasmolysezeit von Hofmeister durch die Gleichung $\Delta G_d = \frac{(C-O) \cdot 60}{C \cdot T}$, wobei C=die Konzentration des Plasmolytiks, O=der osmotische Wert des Objektes in mol Traubenzucker (bei Homogyne etwa 0.650) und T=die Deplasmolysezeit. (Die Berechnung von P'_d , also von P' , gewonnen aus einem Deplasmolysezeitversuch, erfolgt nach der Gleichung

$$P'_d = \frac{120 (C-O)}{T (C+O)}.$$

Obwohl sich nun die »Gewebeschichtung« bei Homogyne alpina in der Anthokyanführung kaum und auch in der Zellform nur wenig ausdrückt ist auch hier der Permeabilitätsunterschied zwischen diesen beiden Schichten aufs deutlichste ausgeprägt. Es zeigt dies, dass diese stets auftretenden Permeabilitätsdifferenzen von der Art des Anthokyan gehaltes der verglichenen Gewebe unabhängig sind und man sie sowohl in solchen Fällen findet, wo die Subepidermis allein gefärbt ist (zB. Taraxacum, obere Zone, vgl. Url 1951), als auch in solchen, wo nur die Epidermis Anthokyan führt (Gentiana Sturmiiana und viele andere Objekte) oder beide Schichten gefärbt sind (Homogyne alpina).

2. Permeabilitätswerte von Epidermen und Subepidermen verschiedener Pflanzen

Es kann als gesichert gelten, dass am Stengel krautiger Pflanzen die Epidermis eine höhere Permeabilität besitzt als die Subepidermis. Den Stengel-epidermen kommt aber nicht nur eine relativ höhere Permeabilität zu, sondern sie sind auch absolut hoch permeabel. Das zeigt eine Betrachtung von Permeabilitätswerten für die Diosmotika Harnstoff und Glycerin, für welche die meisten Erfahrungen vorliegen.

Die in Tabelle 3 angegebenen Werte sind, wenn nicht anders bezeichnet, ΔG -Werte, welche für unsere Fragestellung genügen. Wo in der Literatur die (auf das Gefälle des Diosmotikums von aussen zum Zellsaft reduzierten) Werte der Permeationskonstante P' vorliegen, werden diese angeführt (vgl. dazu Collander und Bärlund 1933, S. 52, Höfler 1934 b, Kreuz 1941).

Aus dieser Zusammenstellung ist zu ersehen, dass ausser den Spitzenwerten hoher Harnstoffpermeabilität bei Epidermen vom »rapiden Harnstofftyp« auch alle anderen untersuchten Epidermen sowohl für Harnstoff wie auch

Tabelle 3.

Objekt	Autor	Epidermis		Subepidermis	
		Harnstoff	Glycerin	Harnstoff	Glycerin
<i>Majanthemum bifolium</i>	Höfler 1934	—	—	0.170	0.055
<i>Muscari racemosum</i>	Hofmeister 1935	—	—	0.251	0.414
<i>Caltha palustris</i> (Blattscheide)	»	—	—	0.065	0.077
<i>Taraxacum officinale</i> (Blattrippe)	»	3.39	0.421	—	—
<i>Gentiana Sturtiana</i>	»	4.0	1.040	—	—
—	Höfler 1936	2.618	—	0.424	—
<i>Curcuma rubescens</i>	Höfler u. Stiegler 1930	—	—	0.048 (P')	0.057 (P')
<i>Ranunculus repens</i>	Markund 1936	—	—	—	—
Glycerintyp	Hofmeister 1938	—	—	—	—
Harnstofftyp	—	—	—	—	—
<i>Lamium maculatum</i>	—	—	—	—	—
trocken	Schmidt 1939	1.04 (P')	3.38 (P')	—	—
feucht	—	1.54 (P')	1.32 (P')	—	—
<i>Valeriana officinalis</i>	—	—	—	0.382 (P')	—
<i>Caltha palustris</i> (Stengel)	Kreuz 1941	0.995 (P')	—	—	—
<i>Mentha aquatica</i>	»	2.340 (P')	—	—	—
<i>Campanula trachelium</i>	»	4.780	—	—	—
<i>Taraxacum officinale</i> (Blattrippe)	»	7.910	—	—	—
<i>Solanum tuberosum</i>	Biehl 1948	2.010	—	—	—
<i>Taraxacum</i> »oben« } (Stengel)	Url 1951	4.0	0.550	—	—
<i>Homogyne alpina</i> 1949	»	0.614	0.381	0.368	0.268
— 1950	»	1.020	0.868	0.140	0.340
— 1951	»	0.330	0.200	0.205	0.053
<i>Aster</i> (Gartenform)	»	0.955	0.436	0.144	0.122
<i>Epilobium hirsutum</i>	»	0.775	0.370	0.180	0.109
<i>Campanula rapunculoides</i>	»	0.302	0.221	0.410	0.246
<i>Gymnadenia conopea</i>	»	12.0 (ΔG _d)	2.15 (ΔG _d)	0.169	0.160
<i>Penstemon hirsutum</i>	»	0.123	0.550	0.90 (ΔG _d)	0.79 (ΔG _d)
<i>Digitalis amandiana</i>	»	1.140	—	—	—
<i>Tradescantia albiflora</i>	»	0.603	—	0.370	—
<i>Daucus carota</i> (Blattstiel)	Fritz 1951 ¹	0.374–0.879 (P' _d)	0.854–1.933 (P' _d)	0.205	—
<i>Vicia faba</i>	»	1.5–4.41 (P' _d)	0.48–1.262 (P' _d)	—	—
—	»	0.895–1.525 (P' _d)	2.507–6.628 (P' _d)	—	—

¹ Die von Fritz gefundenen Werte entstammen einer noch nicht veröffentlichten Dissertation (Wien 1951) und wurden mit freundlicher Genehmigung der Autorin hier angegeben (vgl. Fritz 1952).

für Glycerin eine absolut hohe Permeabilität aufweisen, die stets grösser ist als bei den entsprechenden Subepidermen.

Aber auch die Permeabilitätswerte für die Subepidermen liegen noch recht hoch, wenn man sie mit der bekannt niedrigen Permeabilität von tieferliegenden Parenchymzellen vergleicht (z.B. *Tradescantia*, Hofmeister 1935, Kreuz 1941).

Weitere Beobachtungen haben mir sodann auch gezeigt, dass die Protoplasten des tieferliegenden Grundgewebes durch eine noch wesentlich niedrigere Permeabilität gekennzeichnet sind. Als Beispiel sei ein Versuch an *Taraxacum officinale* angeführt, den ich im September 1951 ausführte. Die Schnitte, die von der unteren Stengelzone (vgl. Url 1951) stammten, waren dicker ausgeführt als jene, die ich sonst für vergleichende Messungen von Epidermis und Subepidermis benützte, um auch für tiefere Parenchymschichten (etwa die 5—6 Zellschichte unter der Epidermis) eine durch Wundnähe nicht gestörte Permeation zu sichern.

Ein solcher Schnitt wurde am 25. September um 13^h49' in eine Lösung von 1.2 mol Glycerin eingelegt.

- 14^h15' Deplasmolysezeitpunkt der Epidermis. (Das entspricht bei einem osmotischen Wert von etwa 0.5 mol Traubenzucker einem ΔG_d von 1.350).
 - 14^h30' Die Subepidermis zeigt noch gute Plasmolysen.
 - 15^h30' Fast alle Zellen der Subepidermis sind deplasmolysiert. (Der Deplasmolysezeit von rund 100 Minuten entspricht ein ΔC_d von 0.350).
 - 17^h15' Das tiefere Parenchym (etwa die 5—6 Schichte) zeigt noch gute Plasmolysen, auch die unter der Subepidermis liegende 3. Zellschichte von aussen ist noch deutlich plasmolysiert.
 - 24^h15' Die tieferen Parenchymschichten sind noch deutlich plasmolysiert. Sie zeigen schöne Systrophien. Die dritte Schicht ist dagegen schon deplasmolysiert.
- Am nächsten Tag 12^h sind sämtliche Zellen des Schnittes deplasmolysiert.

Die Deplasmolysezeit der tieferen Parenchymzellen beträgt somit jedenfalls über 12 Stunden. Schon für diese Zeit errechnet sich ein ΔG_d von unter 0.05. Ähnlich niedrige Permeabilität zeigten auch die tiefsten Parenchymzellen, die an die Markhöhle grenzen. Auch hier ergaben sich (in 1.2 mol Glycerin) Deplasmolysezeiten über 12 Stunden. In der gleichen Grössenordnung liegen diese in 1.2 molarer Harnstofflösung.

Aus solchen Beobachtungen folgt, dass anscheinend ganz allgemein die Epidermis die höchste Permeabilität aufweist, die Subepidermis eine geringere und die tieferen Parenchymlagen die geringste.

Ähnliche Beobachtungen wie an *Taraxacum* machte ich u.a. auch an Zellen des Stengels von *Epilobium hirsutum* und von *Gymnadenia conopsea*. Zu gleichsinnigen Ergebnissen führten Versuche, die ich mit Fettsäureamiden am Stengel von *Physalis Alkekengi* nach der Collanderschen Simultan-

methode (Collander 1949) durchführte, worüber in einer nächsten Mitteilung berichtet werden soll.

Zusammenfassung

Der Angiospermenstengel weist eine auf die verschiedenen Meristemschichten im Vegetationspunkt zurückgehende Gewebeschichtung auf. Diese kann sich in einem verschiedenen Chemismus der Zellsäfte, zB. im Anthokyan-, Flavon-, Gerbstoffgehalt zu erkennen geben (Kasy), sie spiegelt sich aber auch in den Permeabilitätseigenschaften des Protoplasmas wieder. Die Epidermen weisen für verschiedene Diosmotika die höchste Permeabilität auf, die Subepidermen eine geringere und die tiefliegenden Parenchymschichten die weitaus geringste.

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References

- Biebl, R.: Permeabilitätsversuche an der Kartoffelpflanze. — Österr. Bot. Zeitschr. 95: 129. 1948.
- Collander, R.: The permeability of plant protoplasts to small molecules. — *Physiol. Plantarum* 2: 300. 1949.
- & Bärlund, H.: Permeabilitätsstudien an *Chara ceratophylla*. — *Acta Bot. Fennica* 11: 1. 1933.
- & Wikström, B.: Die Permeabilität pflanzlicher Protoplasten für Harnstoff und Alkylharnstoffe. — *Physiol. Plantarum* 2: 235. 1949.
- Elo, J.: Zur Kenntnis der Permeabilitätseigenschaften von *Hippuris vulgaris* L. — *Protoplasma* 32: 3. 1939.
- Fritz, A.: Veränderungen von Plasmaeigenschaften durch Vitalfarbstoffe. — *Sitzungsber. Österr. Akad. Wiss., math. nat. Kl.* 1952, im Druck.
- Höfler, K.: Permeabilitätsbestimmung nach der plasmometrischen Methode. — *Ber. d. deutsch. Bot. Ges.* 36: 414. 1918.
- Permeabilitätsstudien an Stengelzellen von *Majanthemum bifolium*. — *Sitzungsber. Akad. Wiss. Wien, math. nat. Kl. I. Abt.* 143: 213. 1934.
- Permeabilitätsunterschiede in verschiedenen Geweben einer Pflanze. — *Mikrochemie, Molisch-Festschrift*: 224. 1936.
- Spezifische Permeabilitätsreihen verschiedener Zellsorten derselben Pflanze. — *Ber. d. deutsch. Bot. Ges.* 55: 133. 1937.
- Chimärenforschung und Entwicklungsanatomie der Pflanze. — *Sitzungsanzeiger Österr. Akad. Wiss. math. nat. Kl.* vom 8.III.1951: 108.
- & Stiegler, A.: Ein auffälliger Permeabilitätsversuch in Harnstofflösung. — *Ber. d. deutsch. Bot. Ges.* 39: 157. 1921.
- — Permeabilitätsverteilung in verschiedenen Geweben einer Pflanze. — *Protoplasma* 9: 469. 1930.

- Hofmeister, L.: Vergleichende Untersuchungen über spezifische Permeabilitätsreihen. - - Bibliotheca Botanica, Heft 113. 1935.
- Verschiedene Permeabilitätsreihen bei ein und derselben Zellsorte von *Ranunculus repens*. — Jahrb. wiss. Bot. 86: 401. 1938.
- Über die Permeabilitätsbestimmung nach der Deplasmolysezeit. — Sitzungsber. Akad. Wiss. Wien math.-nat. Kl. I Abt. 157: 83. 1948.
- Hurch, H.: Beiträge zur Kenntnis der Permeabilitätsverteilung in verschiedenen Geweben des Blattes. — Beih. Bot. Zentralbl., Abt. I, 50: 211. 1933.
- Kasy, R.: Untersuchungen über Verschiedenheiten der Gewebeschichten krautiger Blütenpflanzen in Beziehung zu den entwicklungsgeschichtlichen Befunden Hans Winklers an Pfropfbastarden. — Sitzungsanzeiger Österr. Akad. Wiss. math.-nat. Kl. 1951: 110.
- Desgleichen. — Sitzungsber. Österr. Akad. Wiss. Wien math.-nat. Kl. im Druck. 1952.
- Kreuz, J.: Der Einfluss von Calcium- und Kaliumsalzen auf die Permeabilität des Protoplasmas für Harnstoff und Glyzerin. — Österr. Bot. Zeitschr. 90: 1. 1941.
- Lange, F.: Vergleichende Untersuchungen über die Blattentwicklung einiger *Solanum-Chimären* und ihrer Elterarten. — Planta. 3: 181. 1927.
- Marklund, G.: Vergleichende Permeabilitätsstudien an pflanzlichen Protoplasten. — Acta Bot. Fennica. 18: 1. 1936.
- Schmidt, H.: Plasmazustand und Wasserhaushalt bei *Lamium maculatum*. — Protoplasma 33: 25. 1939.
- Url, W.: Permeabilitätsverteilung in den Zellen des Stengels von *Taraxacum officinale* und anderer krautiger Pflanzen. — Protoplasma 40: 475. 1951.

Experimental Carbon Dioxide Curves in Photosynthesis

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1. Introduction

Carbon dioxide curves in photosynthesis have since the beginning of this century been used for interpretation of parts of the photosynthetical mechanism. Many of the carbon dioxide curves used for this purpose are, however, quite unreliable. In vol. II, Part 1, chapter 27, of his book on photosynthesis Rabinowitch, 1951, takes an extensive survey of most of the literature and tries to give a summarizing interpretation of the carbon dioxide curves.

Using carbon dioxide curves for investigation of e.g. the carboxylation process in photosynthesis it is important (1) that the curves used are all reliable and (2) that the causes for the deviation of the different reliable curves are adequately analysed.

Despite the valuable contribution of Rabinowitch towards the interpretation of carbon dioxide curves neither point 1 nor point 2 have been satisfactorily treated by him. One of the main reasons for his failure to make most of the carbon dioxide curves available is his oversimplification of the C-assimilation in water plants regarding free CO_2 as the only carbon source.

2. Ability of water plants to assimilate HCO_3^- -ions

Rabinowitch (1951) is aware of some of the modern literature concerning the role of HCO_3^- -ions in the photosynthesis of water plants. But he writes, p. 891: »Pending further analysis concerning the role of carbonate ions

(a question which the above-described experiments have reopened), we will proceed on the old assumption that the rate of photosynthesis is primarily a function of the concentration of the molecular CO_2 in the immediate surroundings of the cells, and that the main effect of the presence of HCO_3^- -ions is to prevent this concentration from depletion during photosynthesis.»

The reason for Rabinowitch not accepting the idea of HCO_3^- -ions being assimilated directly by water plants is without doubt his ignorance of two of the main works on the carbon sources in water plants (Steemann Nielsen 1944 and 1947). In his bibliography he only mentions a preliminary short note in Nature 1946 whereas the main works 1944 (25 pages) and 1947 (71 pages) are unnoticed.

Rabinowitch mentions the observations of Arens (1933, 1936) that in light HCO_3^- -ions are taken up by the lower surface of leaves of aquatic higher plants, while CO_3^{--} or OH^- -ions are set free on the upper surface, thus indicating an uptake of CO_2 from the HCO_3^- -ions in the cells.

Rabinowitch says (p. 888), »that Arens' results are in need of experimental verifications and that, if they prove to be correct, they may be explained (a) by the diffusion of ions through the leaf without penetration into the interior of cells and (b) by cell wall penetration by neutral salt molecules, such as KHCO_3 .» Rabinowitch further mentions that: »in connection with the latter possibility, it would be important to obtain quantitative information on the rate of penetration of bicarbonate as compared to that of free carbon dioxide; conceivably, the observations of Arens could be explained even if the first rate is only one hundredth or one thousandth of the second one, and thus negligible from the point of view of the kinetics of photosynthesis.»

In my 1947 work (p. 39—45) the experiments of Arens were repeated — and on a quantitative basis. It was shown that Arens is right in principle. Only some details of his theory could not stand a critical test.

As the work was published in a Danish periodical not too well-known by plant physiologists, the description of one of the most significant experiments will be repeated here. The apparatus shown in fig. 1 was used. Two round glass cylinders open at both ends and with plane-ground edges each had a content of 15 ml, the area of the opening being 3 cm^2 . A leaf of *Potamogeton lucens* was placed between the two cylinders, which were pressed together by means of rubber bands fastened to metal mountings. A protective layer of paraffin was placed outside. On the morphologically lower side of the leaf was placed natural lake-water (2.7×10^{-3} equiv. HCO_3^-/l), on the upper side redistilled water. The cylinders were closed by means of plane-ground glass plates kept in their places by strong rubber bands. In each of the cylinders a glass rod was placed. The cylinders were rotating in a water

thermostat on a horizontal axis at a rate of 200 revolutions per minute. Thus a vigorous stirring on both sides of the leaf takes place. A beam of light, 24,000 lux) fell directly on the upper side of the leaf. The experimental time was 80 minutes.

In the table below pH and the concentrations of OH^- , CO_3^{--} , and HCO_3^- on the upper side are given before and after the experiment. The concentrations are given in 10^{-5} equiv. per litre.

	pH	eOH^-	eCO_3^{--}	eHCO_3^-
Before	7.9	0.05	0	3
After	10.60	25	37	10

pH on the lower side was at the start 8.20 and at the end of the experiment 8.12. OH^- -ions thus are not released on this side of the leaf during photosynthesis. According to the experiment 1.04×10^{-5} equiv. HCO_3^- (+the corresponding quantity of cations) is taken in on the lower side of the leaf, 0.65×10^{-5} equiv. of which is assimilated in the cells according to the equation $\text{HCO}_3^- = \text{OH}^- + (\text{CO}_2)$. The OH^- -ions are actively transported to the upper side of the leaf and disengaged. Some of the OH^- -ions react with HCO_3^- in the plant or more likely after disengagement on the upper side, as a small quantity of HCO_3^- -ions passes right through the leaf ($\text{OH}^- + \text{HCO}_3^- = \text{CO}_3^{--} + \text{H}_2\text{O}$). A quantity of cations corresponding to the quantities of anions is disengaged on the upper side of the leaf, too.

The assimilation of CO_2 per hour and cm^2 accordingly corresponds to 0.032 mg CO_2 . An identical experiment was made immediately afterwards with the same piece of plant. Here the production of oxygen, however, was measured instead. The assimilation of CO_2 according to this experiment was 0.029 mg CO_2 per hour and cm^2 . It must be mentioned that this is rather a considerable rate of photosynthesis.

This experiment (and the rest — see the 1947 work) shows as clearly as only possible that the leaves of aquatic plants possess a special mechanism for utilizing HCO_3^- -ions in the surrounding water for photosynthesis. The hesitation of Rabinowitch to accept the idea of HCO_3^- -ions participating more directly in photosynthesis thus is not justified.

Furthermore, it should be mentioned that a large number of other observa-

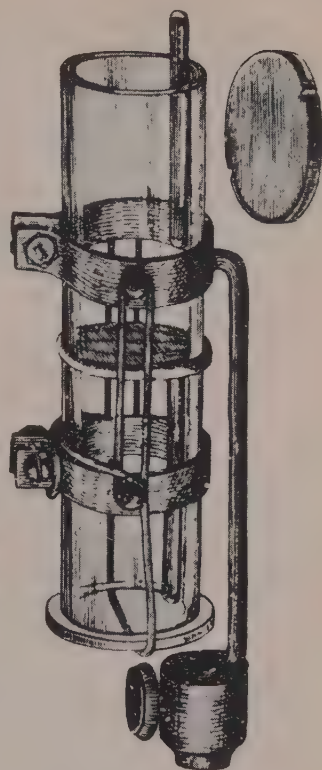


Fig. 1. See text.

tions by the present author — published in the 1944, 1947, and 1951 works — and by Ruttner 1947, 1948, and Österlind 1949, 1950, 1951, in the same way show the direct importance of HCO_3^- -ions in the photosynthesis of many — but not all — water plants.

According to Österlind 1949 the plankton alga *Scenedesmus quadricauda* utilizes bicarbonate better than free carbon dioxide. As, however, photosynthesis in this species seems highly dependable on pH (see further p. 153) this rather improbable statement requires verification.

3. The influence of the concentration of carbon dioxide on photosynthesis

Terrestrial plants are capable of maintaining a considerable photosynthesis in ordinary atmospheric air which only contains about 0.03 per cent. CO_2 by volume. The ability to do so is a *sine qua non* for these plants. In the leaves CO_2 diffuse through the stomata and the intercellular spaces to the cells in which the photosynthesis takes place. As this diffusion takes place in a gas phase, it occurs at an extremely high rate. Only when the CO_2 molecules are to diffuse through the very thin cell walls to the chloroplasts found immediately inside the cell wall, CO_2 must diffuse dissolved in fluids. Such a diffusion takes place at a rate of quite a different and much smaller order of magnitude. The short way for this diffusion (about $1\ \mu$), however, prevents the slow rate from being of absolutely decisive importance. According to all reliable observations on higher terrestrial plants (see Table 27, I, Rabinowitch 1951) the maximum rate of photosynthesis is reached at 0.07 millimoles CO_2 per litre at the most. In unicellular terrestrial algae the maximum rate is obtained at about 0.02 millimoles CO_2 per litre (van den Honert 1930).

In aquatic plants conditions are different, first of all in the submerged leaves of the phanerogams. There are here layers of quite a different order of magnitude across which the CO_2 has to diffuse dissolved in a fluid. The distance from the surface to the chloroplasts farthest away is often about $40\ \mu$ (see Chapter 8, Steemann Nielsen 1947).

It is therefore a matter of course that such plants must require high CO_2 tensions in the water if CO_2 is the only C-source. In my paper just mentioned it was shown that when the water around the plants is strongly stirred the maximum photosynthesis in *Myriophyllum spicatum* and *Potamogeton lucens* is only reached at a CO_2 concentration in the surrounding water of about 0.5—1.0 millimoles per litre, i.e. at a tension of about 1—2 per cent. by volume. At a CO_2 tension of 0.03 per cent., which is found in water being in equilibrium with the atmosphere at about 15°C ., photosynthesis in the

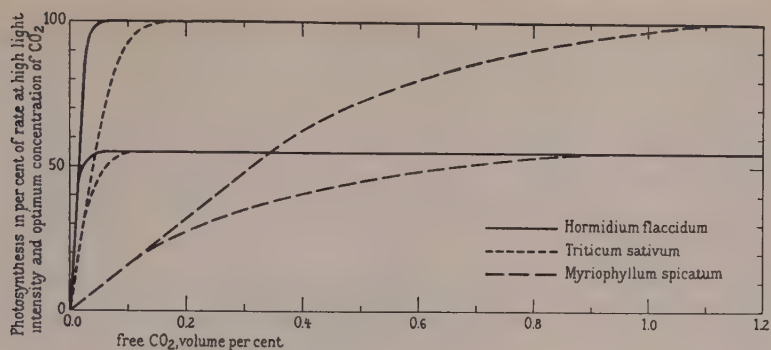


Fig. 2. Three reliable carbon dioxide curves.

plants just mentioned is nearly without any importance if free CO_2 is the only carbon source.

As mentioned in section 2 the phanerogams with submerged leaves can, however, use HCO_3^- -ions besides free CO_2 for the photosynthesis. As the concentration of HCO_3^- in the natural habitat of these plants always is rather high, a low concentration of free CO_2 is without significance.

When in water plants the distance for the carbon source to diffuse is short, it is beforehand to be supposed that rather a low concentration of this source is necessary in order to obtain maximum photosynthesis. For further information see p. 157.

3 sets of curves are drawn together in fig. 2. They present for 3 plant species carbon dioxide curves for both a high light intensity and a light intensity producing at optimum concentration of free CO_2 55 per cent. of the rate of photosynthesis at the high light intensity. The only available carbon source was for all species free CO_2 . The curves for the higher terrestrial plant *Triticum sativum* are taken from Hoover, Johnston, and Brackett 1933. The curves for the terrestrial unicellular alga *Hormidium flaccidum* are from van den Honert 1930 and the curves for the aquatic higher plant *Myriophyllum spicatum* from Steemann Nielsen 1947. All curves are the best ones now available for the different categories of plants.

Whereas the unicellular alga at high light intensity already at 0.05 volume per cent. CO_2 reaches saturation, the higher terrestrial plant does this at 0.15 volume per cent. and the higher aquatic plant not until at 1.1 volume per cent. 22 times as much CO_2 is thus necessary to effect saturation in the aquatic higher plant as in the terrestrial unicellular alga. As already shown diffusion can without difficulty explain this.

It is difficult to say what order of magnitude the concentration of CO_2 should have if a diffusion gradient from the outside to the places in the cells

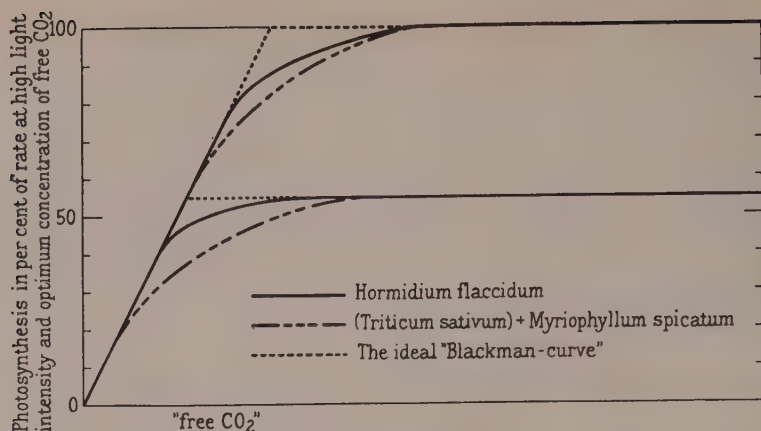


Fig. 3. See text.

where carboxylation takes place could be completely avoided. A better knowledge, i.e. of the nature of carboxylation would be necessary.

If the initial slopes of all curves in fig. 2 are made identical the curves in fig. 3 are obtained. The effect of the different conditions of CO_2 diffusion should in this way to some extent be eliminated. The curves of the three species are now very much alike, the curves for *Triticum* and *Myriophyllum* to such an extent that they cannot easily be drawn separately. The deviation from ideal »Blackman curves» is most pronounced in the two higher plants, without doubt because both the conditions for light and CO_2 are more heterogeneous in the leaves than in a single layer of unicellular algae. It should be mentioned that the carbon dioxide curve for *Fontinalis antipyretica* to be shown in section 5 fits into the scheme, too.

4. Types of unreliable or doubtful carbon dioxide curves

There are a lot of different causes which make carbon dioxide curves more or less unreliable. If all curves where the experimental uncertainty is too pronounced are rejected there still remain several apparently reliable experiments which cannot stand a more thorough criticism.

Harder's experiments (1921) thus seem absolutely convincing. According to these experiments an increase both of the amount of CO_2 and of light, even at very low concentrations of CO_2 causes an increase of the intensity of photosynthesis in *Fontinalis antipyretica*. A curve system of quite another type than that shown in fig. 2 is found. The CO_2 curves for different light intensities have both separated saturation plateaux and distinct initial slopes.

Harder's experiments were made in stagnant water, the bottles with the plants being shaken only once every fifth minute. Steemann Nielsen 1947, fig. 7, showed that *Myriophyllum spicatum* in stagnant water gave precisely the same results as *Fontinalis* in Harder's experiments. »Harder type» (»Bose type» according to Rabinowitch 1951) curve systems are thus only results of using stagnant water. The reason for CO_2 curves for different light intensities not having identical initial slopes in stagnant water is that the convection currents in the solution outside the plants increase with increasing light intensity and thus reduce the external diffusion resistance. Curve systems of the »Harder-Bose type» are thus without importance for kinetic studies in photosynthesis.

Other not quite reliable carbon dioxide curves are represented e.g. by the curves of Smith 1937 and 1938. He investigated the aquatic higher plant *Cabomba caroliniana*. His experimental methodics are in all other respects admirable. Using, however, a manometric technique he employed for obtaining the different concentrations of free CO_2 highly non physiological mixtures of K_2CO_3 and KHCO_3 . The mixtures (no other salts added) ranged from $0.085 \text{ K}_2\text{CO}_3 + 0.015 \text{ KHCO}_3$ to $0 \text{ K}_2\text{CO}_3 + 0.1 \text{ KHCO}_3$ — all concentrations in moles per litre. Concentrations of single salts of this order of magnitude, although often used in experiments, are to a biologist rather absurd. It is probable but not certain that *Cabomba caroliniana* is capable of using HCO_3^- -ions directly for photosynthesis. It is not, of course, inevitable that the submerged leaves of all aquatic higher plants can do this. Some may in this respect behave just like the water moss *Fontinalis aquatica*, which is only able to use free CO_2 (Steemann Nielsen 1946, 1947, Ruttner 1947).

The unnatural and extreme conditions in Smith's experiments, however, give rise to complications. In the carbonate-bicarbonate mixtures producing low concentration of free CO_2 , pH was greater than 10. It is shown by Steemann Nielsen 1947, p. 47, that in *Myriophyllum spicatum* it is completely impossible at pH values of more than about 8 to undertake determination of photosynthesis at high HCO_3^- -concentrations in cases of pure potassium bicarbonate-carbonate solutions. A toxic effect is found which reduces the rate of photosynthesis highly. It is perhaps only the photosynthesis due to HCO_3^- -ions which is affected. In the enormously high concentration of pure potassium carbonates in Smith's experiments the assimilation of HCO_3^- was perhaps completely stopped, the curves being thus real free carbon dioxide curves. The shape of Smith's curves indeed to some extent indicates this. It is, however, at present safest not to utilize these curves for kinetic considerations.

The carbon dioxide curve for the filiform green alga *Cladophora insignis* (Steemann Nielsen 1947, fig. 8) is possibly quite correct. It was, however,

shown in the article mentioned (p. 54—55) that the pH 5.6 used during the experiments in question reduced the rate of photosynthesis considerably in comparison with the rate at higher pH. After about 2 hours photosynthesis nearly ceased. The experiments therefore were of short duration only, thus e.g. not permitting an absolutely safe removal from the cells of reserves of carbon able to be used during photosynthesis.

The carbon dioxide curves of Emerson and Green 1938 do not look reliable. Their method does not make it possible to remove carbon reserves at all. Furthermore they made their experiments with *Chlorella pyrenoidosa* at pH 4.6. According to other observations mentioned below the growth in this species is highly diminished at pH 4.6. These experiments (Österlind 1950) give all in all quite different results.

Emerson and Green admitted an amount of CO_2 to a vessel containing a suspension of *Chlorella pyrenoidosa*, and followed the disappearance of CO_2 by a differential manometer from minute to minute. They could then compute the concentration of the CO_2 remaining at any given time and the corresponding rate of photosynthesis. This method can, however, be used only on condition that the cells do not contain carbon reserves which may be utilized for the photosynthesis. It is rather uncertain whether this condition is fulfilled. I have never seen a species without any appreciable amount of carbon reserves.

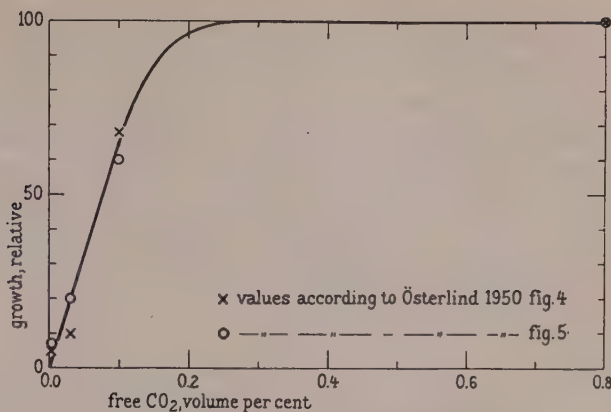
Österlind 1951, too, uses a manometric technique, but he uses a method by which the cells are emptied of carbon reserves before an experiment is started. He gives no complete carbon dioxide curves for the two planktonic algae *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*, but he shows that saturation at pH 4—5 in neither of the species is obtained at a concentration of 0.085 millimoles free CO_2 per litre. There is at this concentration still a proportionality between concentration of carbon dioxide and rate of photosynthesis. This is rather astonishing as saturation should be expected at a somewhat lower concentration in these minute algae, which have only short distances of CO_2 diffusion.

According to growth experiments by Österlind 1950 *Chlorella pyrenoidosa* is highly dependent on pH. At pH 4.2 growth is only about 10 per cent. of that obtained at pH 8.0 when in both cases the concentration of free CO_2 is 0.05 moles per litre. As convincingly shown, this species is unable to utilize HCO_3^- -ions directly.

The experiments on photosynthesis in *Chlorella* by Österlind 1951 were made at low pH (between 4 and 5). At higher pH a much better utilization of CO_2 is to be expected.

According to Whittingham 1949, a thesis not obtainable in Denmark (summary by Rabinowitch 1951 p. 908), the CO_2 demand of *Chlorella pyrenoidosa*

Fig. 4. Carbon dioxide curve (growth) in *Chlorella pyrenoidosa* at pH 8 (according to data by Österlind 1950).



is low. Half saturation of photosynthesis is obtained at 0.0005 to 0.001 millimoles per litre. The experiments were carried out in carbonate buffers. The extreme pH values used for obtaining low concentrations of free CO₂ and the variation of pH from one CO₂-concentration to another must be objected to in this method. In Österlind's growth experiments it was clearly shown that variations in pH have great effects in this species. It is of course possible that *Chlorella pyrenoidosa* at the extremely high pH used by Whittingham can manage photosynthesis at very low concentrations of CO₂. Another explanation is that the strain of *Chlorella pyrenoidosa* used by Whittingham contrary to the strain used by Österlind can utilize HCO₃⁻-ions in photosynthesis.

Österlind's (1951) manometric method seems after all to be the best for obtaining experimental carbon dioxide curves in minute plankton algae. His choice of plant species does not, however, seem to be opportune, as his other object, *Scenedesmus obliquus*, uses HCO₃⁻-ions as carbon source besides free CO₂ and after all is influenced by variations in pH in the same way as *Chlorella pyrenoidosa*. E.g. figure 31, Österlind 1949, is otherwise very difficult to understand. The two circumstances together bring about serious consequences to investigations of the dependence on the concentration of free CO₂.

The most suitable plankton algae in this respect must be such as are normally living in acid water. Both *Chlorella pyrenoidosa* and *Scenedesmus obliquus* are inhabitants of typical alkaline water.

It can thus be said that all carbon dioxide curves in photosynthesis obtained by using minute plankton algae are either unreliable or at any rate doubtful.

The only reliable carbon dioxide curve in these plants is not obtained by

investigating photosynthesis but by investigating growth. Using the results from the growth experiments by Österlind 1950 (his fig. 4 and 5) it is possible to construct carbon dioxide curves. Fig. 4 gives such a curve for pH 8.0. Although experiments at still higher pH possibly would have shown saturation at a somewhat lower concentration of free CO_2 , the present curve is rather instructive. By comparing it with the curve for the terrestrial unicellular alga *Hormidium* (see fig. 1) it is remarkable that saturation in the latter is reached at a concentration of CO_2 of only about one fifth of that in *Chlorella* (see further p. 157).

5. Photosynthesis and concentration of free CO_2 in *Fontinalis antipyretica* and *Fontinalis dalecarlica*

The water moss *Fontinalis antipyretica* L. is unable to utilize HCO_3 in the surrounding water for the photosynthesis as shown by Steemann Nielsen 1946, 1947, and Ruttner 1947. It was shown in my 1947 paper (fig. 6, p. 18) that a concentration of free CO_2 of 0.5 millimoles per litre (i.e. at a tension of about 1 per cent.) was necessary in order to obtain the maximum rate of photosynthesis in *Fontinalis antipyretica*. As the concentration of free CO_2 in the habitat of the plant material for the investigations was always high (order of magnitude 0.3×10^3 mole/l) the high CO_2 demand was not surprising.

Fontinalis antipyretica is, however, found in some Danish lakes with always alkaline (although not extremely alkaline) water (Iversen 1929). According to Sørensen 1948 the pH amplitude for *Fontinalis antipyretica* is 5.5—8.2. The concentration of free CO_2 in alkaline lakes is rather low. It was therefore assumed that the CO_2 demand of the *Fontinalis* plants in these lakes must be less than that found in my investigations of 1947.

The leaves of *Fontinalis antipyretica* are rather thin (about 13 μ). They consist of a single layer of cells only. The distance for the diffusion of CO_2 from the surrounding water to the chloroplasts in, which the photosynthesis takes place is rather short. If the permeability to CO_2 in the *Fontinalis* cells were the same as in the cells of the leaves of higher water plants, already rather a low concentration of free CO_2 in the surrounding water should be able to make a considerable rate of photosynthesis possible.

It is, of course, possible that *Fontinalis antipyretica* in alkaline lakes represents another physiological type than the specimens found in water with a higher concentration of free CO_2 . Before investigating this it was, however, found opportune to reinvestigate the CO_2 demand of the *Fontinalis* plants from the same locality as in 1947. The main reason for doing so was

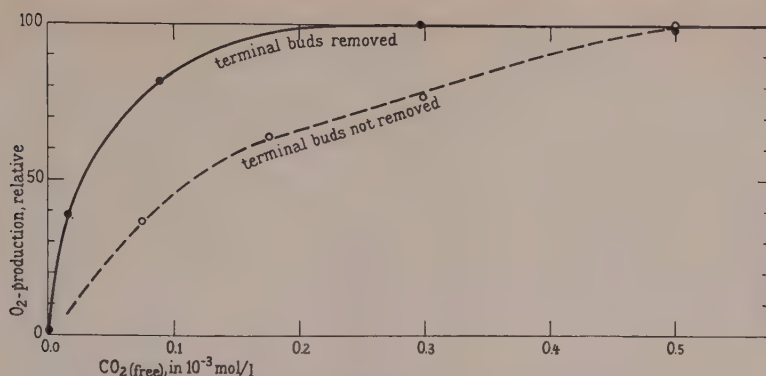


Fig. 5. *Fontinalis antipyretica*. Carbon dioxide curves at 15,000 lux, 22° C., pH 4.4.

some observations on the morphological aspects of *Fontinalis antipyretica* from different localities.

Whereas the shoots from alkaline lakes have rather long internodes and a rather small terminal bud, the specimens from the CO₂-locality had much shorter internodes and a much bigger terminal bud. A great many fully developed leaves form a compact bulk at the head of the shoot. At the investigations of the CO₂ demand in 1947 the terminal buds were not removed. As furthermore rather short shoots were used, a considerable number of the photosynthesizing cells were found in the compact bulk of leaves. The diffusion conditions for CO₂, therefore, must be badly affected because of the diffusion channels being much prolonged.

Some new experiments on the CO₂ demand were therefore made, using shoots with the terminal buds removed. Specimens from the same locality as in the former investigations were used. Fig. 5 gives these experiments graphically (full line). The experiments from 1947 are given at the same time (broken line). The two curves clearly show the significance of removing the terminal bud. Whereas maximum photosynthesis was not obtained until at a CO₂ concentration of 0.5 millimoles per litre in the material with the terminal buds present, this was already the case at about 0.25 millimoles per litre when the terminal buds were removed. At lower concentrations of CO₂ the difference between the two plant materials is still more significant. At a concentration of free CO₂ of 0.03 millimoles per litre only 15 per cent. of the maximum rate of photosynthesis is obtained if the terminal buds are not removed. If they are removed, 52 per cent. of the maximum rate is found. In the slightly alkaline lakes where *Fontinalis antipyretica* is found, the concentration of free CO₂ is seldom less than 0.03 millimoles per litre. Thus the CO₂ conditions in these lakes are not too bad. As mentioned above,

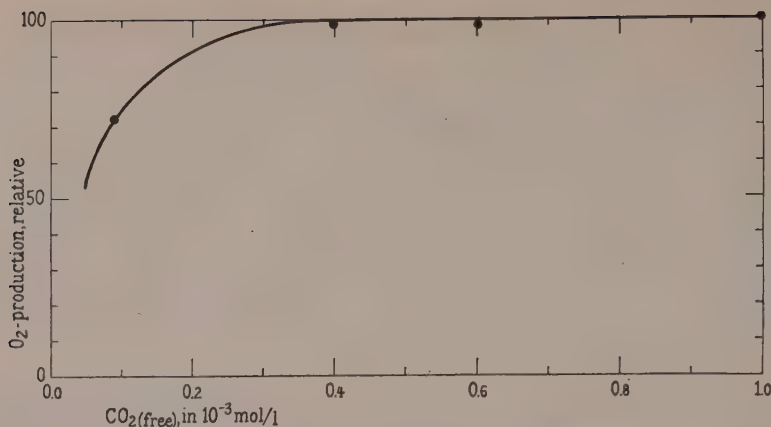


Fig. 6. *Fontinalis dalecarlica*. Carbon dioxide curve at 15.600 lux, 17° C., pH 4.5.

Fontinalis antipyretica is not found in extremely alkaline lakes where pH during the summer rises to high values (about 9).

The experiments given in fig. 5 show that the demand for free CO₂ in *Fontinalis antipyretica* is far from being as high as I thought formerly. Still the demand is perhaps a little higher than should be expected (see below, p. 157).

The ecological distribution of *Fontinalis dalecarlica* Schimp. differs clearly from that of *Fontinalis antipyretica*. Iversen (1928), who investigated the distribution of the two species in Denmark, found the last-mentioned species in alkaline, neutral, and acid water. *Fontinalis dalecarlica* was found only in acid water. According to Sørensen (1948) the pH amplitude for *Fontinalis dalecarlica* is 4.2—6.6 (8.6 is a misprint). For *Fontinalis antipyretica* it is 5.5—8.2.

Fontinalis dalecarlica was collected from a lake, Grib Sø, in North Zealand in June 1949, and experiments on the demand of free CO₂ for the photosynthesis were made. The terminal buds were removed. The leaves were 7 μ thick. The methodics were the same as those used for *Fontinalis antipyretica*. pH was 4.5 in the water used for the experiments. At this hydrogen ion concentration only free CO₂ is found. The temperature was 17° C., the illumination 15.600 lux.

The curve illustrating the experiments (fig. 6) is nearly identical to that obtained with *Fontinalis antipyretica* (cp. fig. 5). The maximum photosynthesis is obtained at a CO₂ concentration of about 0.3 millimoles per litre — i.e. at a CO₂ tension of about 0.6 per cent. by volume.

The different ecological behaviour of the two *Fontinalis* species thus is hardly caused by a different CO₂ demand for the photosynthesis.

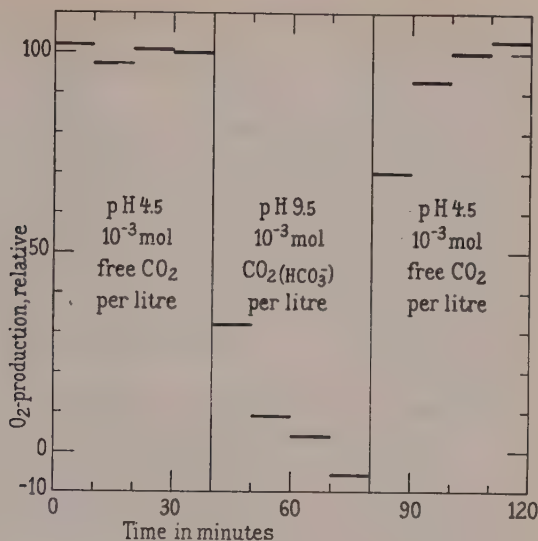


Fig. 7. *Fontinalis dalecarlica* (see text).

In both *Fontinalis* species investigated saturation thus is not obtained until at rather a high concentration of free CO_2 in the solution surrounding the leaves, although these have only one layer of cells. As stated above (p. 154), a similar high CO_2 demand is found in the minute plankton alga *Chlorella pyrenoidosa*.

Two possible explanations can be mentioned:

(1) Layers of water adhering to the plants set up a considerable diffusion potential outside the plant although the experimental water is vigorously stirred.

(2) The permeability to free carbon dioxide is low in aquatic plants unable to use HCO_3^- -ions in the surrounding water for photosynthesis.

Against (1) speaks the fact that saturation in the plankton alga *Scenedesmus quadricauda* according to Österlind is obtained at very low concentrations of bicarbonate, most likely as low as 0.01 millimole per litre (1951 p. 251). Experiments using free CO_2 as carbon source are not of much value in this species, as the low pH used under these circumstances, as mentioned above, involves serious complications. Diffusion potentials outside the plant must, however, occur in equal dimensions for HCO_3^- -ions and for free CO_2 . External diffusion potentials can therefore scarcely be the cause of the high CO_2 demand in *Chlorella* and *Fontinalis*. A low permeability to free CO_2 in these species is more likely.

It is not, however, at present possible with certainty to explain a connec-

tion between the low permeability of free CO_2 and the lack of ability to assimilate HCO_3^- -ions although the latter may be explained as caused by impermeability to this ion.

It remains to demonstrate the inability to use HCO_3^- -ions for photosynthesis also in the *Fontinalis* species *F. dalecarlica*. The experiments illustrated in fig. 7 demonstrate this clearly. Although the HCO_3^- concentration in the experiments at pH 9.5 is high, no photosynthesis is demonstrable after the carbon reserves in the cells are used. The carbon reserves in *Fontinalis dalecarlica* are rather small. After 30 minutes in strong light they have completely disappeared. This is in accordance with the results obtained in *Fontinalis antipyretica*.

The lack of photosynthesis at pH 9.5 is not a direct result of the high OH^- concentration damaging the plants. When changing to pH 4.5 photosynthesis starts immediately. Already after 10 minutes the rate is at maximum. In another article in this journal it will be shown that *Fontinalis dalecarlica* will stand a much higher pH than 9.5, at least for short periods.

Summary

When using carbon dioxide curves of aquatic plants for kinetic studies it is necessary to consider the ability to use HCO_3^- -ions as a direct carbon source in the photosynthesis. The mechanism of HCO_3^- utilization in the photosynthesis of aquatic higher plants is demonstrated with the aid of a quantitative experiment showing uptake of HCO_3^- -ions together with the corresponding cations from the lower side of a *Potamogeton* leaf and disengagement of OH^- -ions from the upper side together with the corresponding cations.

The importance of the diffusion potentials for CO_2 inside the plants is demonstrated.

It is shown that a very considerable number of the carbon dioxide curves published are not reliable. Three reliable curves are compared.

Carbon dioxide curves for the two *Fontinalis* species *F. antipyretica* and *F. dalecarlica* are also published. The importance of removing the terminal buds in these species is demonstrated. It is shown that *F. dalecarlica* just like *F. antipyretica* is unable to utilize HCO_3^- -ions in the surrounding water for photosynthesis.

It is rendered probable that the permeability to free CO_2 is low in all investigated aquatic plant species without any ability to use HCO_3^- -ions in photosynthesis. There may be some connection between the relatively low permeability to free CO_2 and the impermeability to HCO_3^- -ions.

References

- Arens, K.: Physiologisch polarisierter Massenaustausch und Photosynthese bei submersen Wasserpflanzen. I. — *Planta* 20: 621. 1933.
- Physiologisch polarisierter Massenaustausch und Photosynthese bei submersen Wasserpflanzen. II. Die $\text{Ca}(\text{HCO}_3)_2$ -Assimilation. — *Jahrb. f. wissensch. Botan.* 83: 513. 1936 a.
- Photosynthese von Wasserpflanzen in Kaliumbikarbonatlösungen. — *Ibidem*, 83: 561. 1936 b.
- Emerson, R. and Green, L.: Effect of Hydrogen-Ion Concentration on *Chlorella* Photosynthesis. — *Plant Physiology* 13: 157. 1938.
- Harder, R.: Kritische Versuche zu Blackmans Theorie der »begrenzenden Faktoren« bei der Kohlensäureassimilation. — *Jahrb. f. wissensch. Botan.* 60: 531. 1921.
- Honert, T. H. van den: Carbon dioxide assimilation and limiting factors. — *Rec. trav. bot. néerl.* 27. 1930.
- Hoover, W. H., Johnston, E. S. and Brackett, F. S.: Carbon Dioxide Assimilation in a Higher Plant. — *Smithsonian Inst. Publ. Misc. Collections*, 87, No. 16. 1933.
- Iversen, J.: Studien über pH-Verhältnisse dänischer Gewässer und ihren Einfluss auf die Hydrophyten-Vegetation. — *Bot. Tidsskr.* 40: 277. 1929.
- Rabinowitch, E.: Photosynthesis and related Processes. Vol. II. Part 1. New York. 1951.
- Ruttner, F.: Zur Frage der Karbonatassimilation der Wasserpflanzen. Eine vergleichende Untersuchung. 1. Teil: Die beiden Haupttypen der Kohlenstoffaufnahme. — *Österr. Botan. Zeitschr.* 94: 265. 1947.
- Zur Frage der Karbonatassimilation der Wasserpflanzen. Eine vergleichende Untersuchung. 2. Teil: Das Verhalten von *Elodea canadensis* und *Fontinalis antipyretica* in Lösungen von Natrium- bzw. Kaliumbikarbonat. — *Ibidem* 95: 208. 1948.
- Smith, E. L.: The Influence of Light and Carbon Dioxide on Photosynthesis. — *The Journ. of Gener. Physiology* 20: 807. 1937.
- Limiting Factors in Photosynthesis: Light and Carbon Dioxide. — *Ibidem* 22: 21. 1938.
- Sørensen, H.: Studies on the Ecology of Danish Water- and Bog Mosses. — *Dansk Bot. Ark.* 12, Nr. 10. 1948.
- Steemann Nielsen, E.: Dependence of Freshwater Plants on Quantity of Carbon Dioxide and Hydrogen Ion Concentration. Illustrated through Experimental Investigations. — *Dansk Botan. Ark.* 11, Nr. 8. 1944.
- Carbon Sources in the Photosynthesis of Aquatic Plants. — *Nature* 158: 594. 1946.
- Photosynthesis of Aquatic Plants with special Reference to the Carbon-Sources. — *Dansk Botan. Ark.* 12, Nr. 8. 1947.
- Passive and Active Ion Transport during Photosynthesis in Water Plants. — *Physiol. Plant.* 4: 189. 1951.
- Whittingham, C. P.: Thesis, Cambridge Univ. 1949.
- Österlind, S.: Growth Conditions of the Alga *Scenedesmus quadricauda* with special reference to the Inorganic Carbon Sources. — *Symb. Botan. Upsaliens.* 10, Nr. 3. 1949.
- Inorganic Carbon Sources of Green Algae. I. Growth Experiments with *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*. — *Physiol. Plant.* 3: 353. 1950.
- Inorganic Carbon Sources of Green Algae. III. Measurements of Photosynthesis in *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*. — *Ibidem* 4: 242. 1951.

Effects of Different Silicon Concentrations on the Growth of Diatoms

By

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Introduction

Studies on the dependence of plant growth on the supply of silicon have previously been connected with great difficulties. A major one arose from the fact that the containers used for cultivation even when the hardest kinds of glass were employed, gave off silicon to the nutrient solutions. This difficulty was overcome by Richter (1904 and 1906), who used glass flasks with an inside coating of paraffin and thus was the first to show that silicon is a substance necessary for the growth of diatoms. The method is, however, very difficult to use owing to the instability of the paraffin coating. King and Davidson (1933) used glass containers with a coating of bakelite varnish.

A second and smaller difficulty presents the preparation of redistilled water free of silicon. This difficulty was also overcome by Richter, as his water did not come into contact with glass.

Because of these difficulties our knowledge of the growth of diatoms at low silicon concentrations is negligible. At higher silicon concentrations, the amounts given off from the glass are of but small importance. If the growth in the cultures is intense, the silicon content of the nutrient solution will soon decrease considerably, since silicon is given off from the glass more slowly than it is absorbed by the diatoms (King and Davidson 1933). I have observed the same conditions in many of my cultures in glass containers.

The dependence of the diatoms on the content of silicon at higher con-

centrations has been studied by Brieger (1924) and Chu (1942), who endeavoured to determine the optimum concentrations for the species in question.

Recently a vast improvement in technique was made possible through the appearance of the various plastics and of the synthetic ion exchange resins. In the following is described a series of experiments with *Nitzschia palea* cultivated at various silicon concentrations in plastic containers.

Methods

Containers. For the experiments were used containers, 6.5 cm in height and 5.5 cm in diameter, provided with lids. They were made of polystyrene, a substance which is very resistant to most chemical solutions. Thus it is not attacked by strong or weak acids and alkalis, nor by aliphatic hydrocarbons and alcohol. It has a low permeability to water and a light transmission of 90 per cent. Several parallel experiments have shown that in the plastic containers there is as good growth as in glass flasks, and that the polystyrene thus does not give off any substance with a toxic effect.

Polystyrene becomes plastic at 77–80° C, so that the containers with the nutrient solution cannot be sterilized in the usual way. Instead two successive heatings to 70–75° C for two hours at an interval of 24 hours have been used.

The water. In ordinary distilled water which had not been in contact with glass, the cations present (particularly Pb and Cu) were absorbed on a cation exchanger (Amberlite I R-100) in an apparatus consisting exclusively of polystyrene.

Nutrient solution. A modification of Rodhe's Culture solution VIII (Rodhe 1948) was used:

Ca(NO ₃) ₂	60.0	mg/l
MgSO ₄	5.0	»
MnSO ₄	0.03	»
Na ₂ HPO ₄	5.0	»
KHCO ₃	200.0	»
Ferric citrate-citric acid	1.0+1.0	mg/l
Soil extract	5	ml/l

The last four components were added after the heating of the culture solutions to 70° C. Na₂HPO₄ and ferric citrate-citric acid were kept in plastic containers (like the other stock solutions) and sterilized in the same way as the culture solution itself. KHCO₃ was autoclaved in a pyrex bottle with a patent stopper, and a strong stock solution of 100 mg KHCO₃ per ml was used in order to have as little silicon added to the nutrient solution as possible. Determinations in 1 liter distilled water to which was added the quantity of KHCO₃ used in the nutrient solution showed no presence of silicon. This was true even when 10 times this quantity was used. The soil extract was kept and autoclaved in a pyrex flask.

Silicon solution. As it has not yet been possible for me to obtain sodium silicate pro analysis, I have had to purify a pure sodium silicate, as a series of experiments with pure sodium silicate showed a distinct toxic effect at a concentration of

10 mg/l Si. 400 mg pure anhydrous Na_2SiO_3 was dissolved in ion exchange water and precipitated with HCl. The silicic acid was washed several times on a filter with warm ion exchange water and then heated to constant weight in a platinum crucible. 1 g Na_2CO_3 , anhydrous p.a., was added and the mixture was fused by means of a Meker burner. A stock solution of the concentration 100 mg Si per l was made. The excess Na_2CO_3 was neutralized with HCl, the stock solution being titrated down to pH=8.

Illumination and temperature. The experiments were made in two water thermostats, each having room for 20 plastic containers. In the series of experiments mentioned a temperature of 20° C was used in one water thermostat and a temperature of 25° in the other. The plastic containers were illuminated from above by means of six tubular fluorescent lamps (20 W, daylight), providing an illumination of 3500 lux in the experiments. A constant illumination was maintained during the whole period of the experiments.

Determination of chlorophyll. The growth was determined partly through the determination of chlorophyll, partly by counting the cells. As the diatom used, *Nitzschia palea*, grows on the bottom of the containers and sticks firmly to it, it is easy to prepare a cellfree chlorophyll extract. After the culture solution was decanted, 10 ml methyl alcohol was added to each container, and the containers were heated for 1 minute in a water bath of about 80° C, after which the extract was filtered through a glass filter. The extinction values of the chlorophyll solutions were determined photometrically with an »EEL» Portable Colorimeter, using a red filter (205 red, mean wave length of transmission 660 mμ).

The quantities of chlorophyll are given as extinction values (see Rodhe 1948 p. 11), the extinction (e) being defined by the formula

$$e = \log \frac{I_0}{I},$$

I_0 in the case of chlorophyll determinations being the deflection of the galvanometer when there is pure methyl alcohol in the test tube, and I the deflection when the chlorophyll extract in question is in the test tube.

For measurements with the »EEL» Colorimeter a test tube containing pure methyl alcohol is first placed in the colorimeter, and the galvanometer is by means of the variable shutter adjusted to zero. After substituting the chlorophyll sample for the methyl alcohol, the extinction is read directly from the logarithmic scale of the galvanometer.

In the case of determinations of silicon and phosphate, in which the »EEL» Colorimeter was also applied, the examined nutrient solution without addition of reagents was used for the adjustment of the galvanometer instead of pure methyl alcohol. Still, the same amount of acid was added to the nutrient solutions as was found in the reagents in the case of the determinations in question, in order that possible organisms, proteins, etc., could be precipitated in the same way in the two test tubes to be compared.

With the »EEL» Colorimeter the chlorophyll determination gives an accuracy of ± 0.1 expressed as extinction values.

Counting of the diatom cells. The diatoms were detached from the bottom of the plastic container by being left for 24 hours with about 5 ml conc. H_2SO_4 , after which the bottom was rubbed thoroughly with a flat plastic spatula. After suitable dilutions of the sulphuric acid with the diatoms, a dry preparation was made from 0.2 ml

solution, in which the number of diatom cells was counted. The dilutions were made in such a way that each preparation contained from 200 to 500 cells. The number of cells per culture then was calculated on the basis of the dilutions. At least two counts were made of each culture.

As conc. H_2SO_4 is rather inconvenient to handle, I have tried to detach the diatoms with other chemicals such as diluted H_2SO_4 , diluted HCl , and alcohol. Examinations of the bottoms of the plastic containers through an inverted microscope after the treatments show, however, that only conc. H_2SO_4 gives a satisfactory result.

By a number of parallel experiments it was investigated whether the treatment with sulphuric acid gives rise to changes in the plastic containers which influence the growth of the diatoms, and it was shown that the growth in containers treated and not treated is identical. Even in containers treated with conc. H_2SO_4 for a month there is the same growth as in containers not treated.

Determinations of silicon. The contents of silicon in the solutions were determined according to Diénert and Wandenbulcke's colorimetric method (D. and W. 1923). 10 ml-samples were used to which were added 4 drops of 5 per cent H_2SO_4 and 0.1 ml ammonium molybdate (10 g ammonium molybdate in 100 ml water). The extinction values of the solutions were determined photometrically with the »EEL» Colorimeter, using Chance filter: OB 10 (wave-length range 330—510 m μ). According to Robinson and Spoor (1936) this filter gives a maximum absorption at the colorimetric determination of silicon. Calibration was made with a silicon solution prepared from the above-mentioned stock solution of 100 mg Si per l (see Fig. 4). Solutions of concentrations higher than 15 mg Si per l should be diluted, as the amount of ammonium molybdate added is insufficient for higher concentrations (see Thayer 1930). Solutions containing less than 0.2 mg Si per l should be evaporated to one fifth to one tenth the initial volume to give reasonable results. For silicon concentrations below 1000 μg Si per l the method gives an accuracy of ± 50 μg Si per l. Between 1 and 15 mg Si per l the accuracy is ± 100 μg Si per l.

Determination of phosphate. The content of phosphate was determined according to Denigès' colorimetric method (D. 1920), as described in Wattenberg 1937 p. 11. 100 ml samples were used to which were added 1 ml reagent I and 0.15 ml reagent II. Reagent I: 1 part of ammonium molybdate (10 g ammonium molybdate in 100 ml distilled H_2O) + 3 parts of 50 per cent H_2SO_4 . Reagent II: 0.125 g stannous chloride in 25 ml HCl (1 part of conc. HCl + 10 parts of distilled H_2O). The extinction values of the solutions were determined photometrically with the »EEL» Colorimeter with the red filter (205 red). A calibration curve was drawn on the basis of a standard phosphate solution containing 0.1 g P/l (0.439 g KH_2PO_4 in 1 l distilled H_2O). The solutions were diluted 10 times for concentrations higher than 100 μg P per l. The determination of phosphate gives an accuracy of ± 2 μg P per l for concentrations up to 100 μg P per l.

Stock culture of diatoms. The stock culture of *Nitzschia palea* used was inoculated from the surface of a percolating filter of a sewage work. It was cultivated in the above-mentioned nutrient solution in pyrex flasks. It was not completely free of bacteria. About 500 diatom cells were added to each plastic container.

Experiments

Experiments were made under the following conditions: (1) Containers: plastic containers (with lids) of polystyrene. (2) Constant Illumination: 3500 lux. (3) Temperature: Series I 25° C, series II 20°. (4) Ion exchange water. (5) Rodhe's culture solution VIII. (6) pH=8.5 in all cultures. (7) Inoculation: *Nitzschia palea* (Kütz.) W. Smith, about 500 cells to each culture. (8) Silicon concentrations: 10, 5, 1, 0.5, 0.1 mg Si per l. (9) Duration of the experiments: 3 weeks (25/6—17/7 1951).

The experimental conditions appear from the survey above. Four parallel cultures were used in each single experiment with 50 ml nutrient solution in each plastic container. The growth was determined partly by the cell count, partly by determinations of chlorophyll. The table shows the determinations

Table 1. *Growth of Nitzschia palea at different Si-concentrations.*

Temperature	I	25°		II	20°	
Concentration of Si mg/l	No.	Chlorophyll content, extinction value	Number of cells	No.	Chlorophyll content, extinction value	Number of cells
10	1	6.3	11.450.000	21	4.3	7.838.000
	2	3.2	4.413.000	22	4.3	9.988.000
	3	3.5	4.906.000	23	7.1	17.000.000
	4	3.2	5.375.000	24	8.5	15.875.000
average		4.1	6.536.000		6.1	12.800.000
5	5	6.1	7.760.000	25	5.3	14.188.000
	6	4.5	11.472.000	26	6.5	12.031.000
	7	4.4	11.256.000	27	6.5	11.781.000
	8	2.4	3.583.000	28	6.3	12.594.000
average		4.4	8.583.000		6.2	12.649.000
1	9	2.1	414.000	29	1.8	3.788.000
	10	1.9	2.150.000	30	1.8	3.700.000
	11	1.7	2.656.000	31	2.1	3.163.000
	12	1.3	1.944.000	32	1.5	1.275.000
average		1.8	1.791.000		1.8	2.982.000
0.5	13	1.2	96.000	33	0.8	236.000
	14	0.8	170.000	34	1.1	294.000
	15	0.8	535.000	35	1.1	293.000
	16	0.7	405.000	36	1.2	295.000
average		0.9	302.000		1.1	280.000
0.1	17	0.0	4.500	37	0.1	15.000
	18	0.0	700	38	0.1	17.250
	19	0.0	1.250	39	0.0	33.600
	20	0.0	2.800	40	0.1	17.500
average		0.0	2.300		0.1	20.800

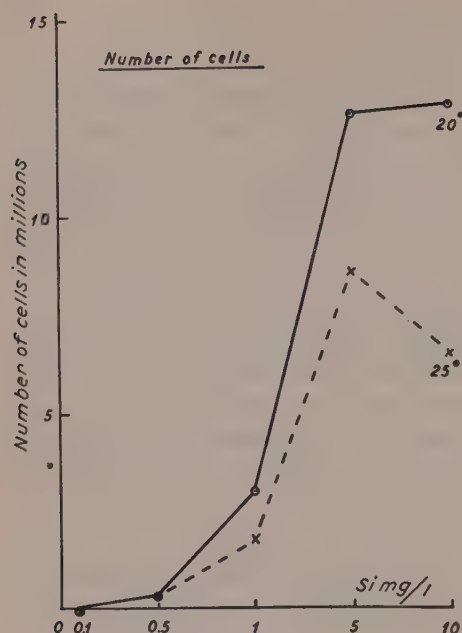


Fig. 1. Growth of *Nitzschia palea* at various Si-concentrations. At 1 mg Si per l the scale of the abscissa is changed.

of chlorophyll and the cell counts in the 40 single experiments. In Figs. 1 and 2 are plotted the average values of growth (cell counts and amounts of chlorophyll, respectively) against Si-concentration for four parallel cultures at two different temperatures.

It is seen that there is a distinct proportionality between the content of chlorophyll and the Si-concentration (Fig. 2), and it appears from Fig. 1 that the number of cells increases with increasing Si-concentration. This is evident at the concentrations 0.1, 0.5, 1, and 5, whereas the growth at 10 mg Si per l is approximately the same as at 5 mg Si per l. It appears from the figures that the average growth is greater at 5 than at 10 mg Si per l at 25°. However, it appears from Table 1 that there is a great variation of the single cultures, and that thus the difference in the averages cannot be considered an absolute difference between the two experiments.

The very great variation in the single cultures at 25° is not found at 10 mg Si per l only, but at all the five concentrations investigated. No doubt, this indicates that a temperature of 25° is the upper limit of what may give good growth of the species of diatom investigated. At 20° it is seen that the variation between the single cultures at the five different Si-concentrations is considerably smaller.

A determination of the silicon content of the culture solutions at the termination of the experiments shows that approximately the same Si-

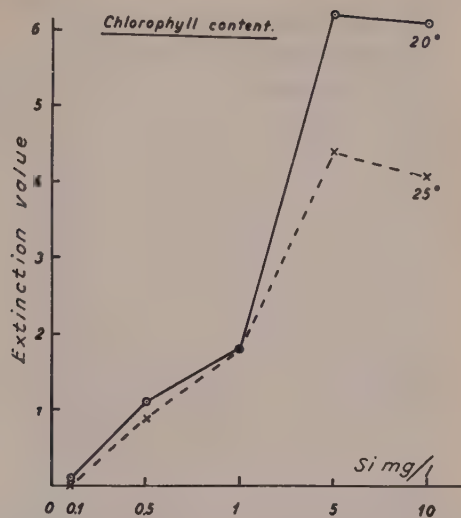


Fig. 2. Growth of *Nitzschia palea* at various Si-concentrations. The abscissa is as in Fig. 1.

absorption took place at the concentrations 5 and 10 mg Si per l. At 20° about 5 mg Si per l was absorbed in both cases and at 25° about 4 mg Si per l. In longer lasting experiments a greater production will ensue at 10 mg Si per l, as at the termination of the experiments only a small amount of silicon was left in the culture solution in the series with 5 mg Si per l, whereas in the series with 10 mg Si per l still about half the initial amount was present.

Furthermore, it appears that the absorption of Si takes place faster at 20° than at 25°. This is evident at the Si-concentrations 5 and 10 mg Si per l. At the lower concentrations there were in all experiments so little silicon left that further growth was prevented, and there was approximately the same production at the two different temperatures.

Influence of Phosphate Concentration on the Determination of Silicon

In the experiments mentioned the silicon concentrations were not kept constant. Therefore it was of great interest to have the concentrations at the end of the experiments determined. However, there proved to be certain difficulties in such determinations as the high content of phosphate in the nutrient solutions influenced the determination of silicon. Phosphate, like silicate, forms a yellow compound with ammonium molybdate, so that any phosphate present will cause an increase in the extinction values measured.

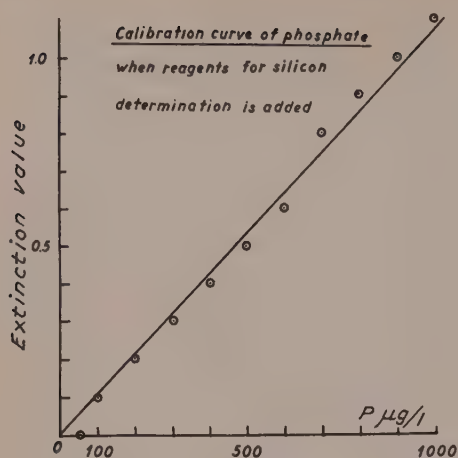


Fig. 3. Calibration curve of phosphate when reagents for silicon determination is added. Filter Chance OB 10.

The method used for the determination of Si (see p. 164) is intended for determinations in natural waters. The amount of phosphate contained in unpolluted freshwater and sea water is so slight that it does not influence the determination of Si, as pointed out by Atkins (1926 p. 90). He writes: »It has been established that the presence of phosphate in the water introduces no error into the silicate estimation, for 0.5 mg per l of P_2O_5 (=about 110 µg P per l) yields at tint equivalent to 0.08 mg per l silica, whereas only in the deeper ocean water is as much as 0.05 mg per l of P_2O_5 found». The effects of the presence of phosphate and iron on the colorimetric determination of silicon have been more closely studied by Thayer (1930). In his experiments comparatively large amounts of phosphate and iron were used, so that they do not sufficiently throw light on the critical spheres which are of interest here.

Fig. 3 presents a calibration curve showing the extinction values of various phosphate solutions free of silicon when the same reagents are added as for determinations of silicon (see p. 164). Fig. 4 for comparison shows a calibration curve for various solutions of silicon. It appears from Fig. 4 that 100 µg P per l gives the same extinction values as 50 µg Si per l. The nutrient solution used contains about 1000 µg P per l, which gives an extinction value corresponding to 0.55 mg Si per l. At the termination of the experiments there were from about 400 to 600 µg P per l in the nutrient solutions corresponding to an amount of silicon of 0.2–0.3 mg Si per l. The amount of phosphate was determined according to Denigès' colorimetric method (see p. 164). It should be noted that whereas the presence of phosphate influences the determinations of silicon with the method used, the presence of silicon does *not* influence the determinations of phosphate with Denigès' method.

The amount of silicon left at the termination of the experiments at the

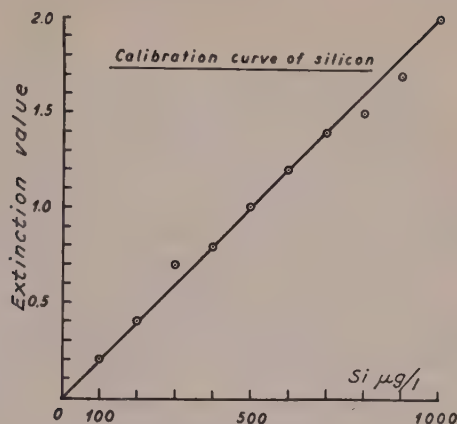


Fig. 4. Calibration curve of silicon. Filter as in Fig. 3.

smaller Si-concentrations is probably about 0.05 mg Si per l. In order to determine such small amounts of silicon with the method used it is necessary to evaporate to about one fifth to one tenth the initial volume, thus causing an increase in the phosphate concentration (some evaporations to one tenth gave a content of phosphate of about 1.5 mg P per l, the extinction value of which corresponds to that of 0.8 mg Si per l; some of the phosphate was precipitated during the evaporation). Thus it is not possible, with the phosphate concentrations used, to obtain reproducible results for such small amounts of silicon.

Accordingly the lowest silicon values of King and Davidson (0.15 mg/l SiO_2) may be too high because of the great content of phosphate in the nutrient solution used (about 4 mg P per l).

As shown by Thayer (1930) the presence of iron along with phosphate also influences the determination of silicon. The amount used here, 0.18 mg Fe per l, gives an extinction value of 0.2, i.e. the same value as 0.1 mg Si per l. The magnitude of the iron concentration at the termination of the experiments was not determined, and the quantity left may be of no importance.

Soil extract, too, has proved to introduce errors into silicon determinations. The small amount used here gives an increase of the extinction value of 0.1, corresponding to 0.05 mg Si per l.

Thus, if the silicon concentration at the termination of the experiments is to be determined with a reasonable accuracy smaller amounts of phosphate should be used than has here been the case. A later series of experiments with different concentrations of phosphate showed that 500 and 200 µg P per l give just as good growth as 1000 µg P per l. Furthermore, the use of soil extract should be avoided, and possibly a lower iron concentration should be used in the nutrient solution.

Summary

Series of experiments at respectively 20° and 25° C. with *Nitzschia palea* at the Si-concentrations 0.1, 0.5, 1, 5, and 10 mg Si per l and constant illumination are described. The diatoms were cultivated in plastic (polystyrene) containers. The growth was determined in terms of either number of cells per culture, or amounts of chlorophyll content expressed as extinction values. The results of the experiments appear from Table 1, and from Figs. 1 and 2. The chlorophyll content as well as the number of cells increase with increasing Si-concentration. The absorption of Si takes place more quickly at 20° than at 25°.

The influence of the phosphate concentration on the determinations of Si was investigated as appears from Fig. 3.

References

- Atkins, W. R. G.: Seasonal changes in the silica content of natural waters in relation to the phytoplankton. — *Journ. Mar. Biol. As.* 14: 89. 1926.
- Brieger, F.: Über den Siliciumstoffwechsel der Diatomeen. — *Ber. deutsch. Bot. Gesell.* 42: 347. 1924.
- Chu, S. P.: The influence of the mineral composition of the medium on the growth of planktonic algae. I. Methods and culture media. — *J. Ecol.* 30: 284. 1942.
- Denigès, G.: Réaction de coloration extrêmement sensible des phosphates et des arseniates. — *C. R. Acad. des Sciences, Paris*, 171: 802. 1920.
- Diéner, F. & Wandenbulcke, F.: Sur le dosage de la silice dans les eaux. — *C. R. Acad. des Sciences, Paris*, 176: 1478. 1923.
- King, J. & Davidson, V.: The biochemistry of silica acid. IV. Relation of silica to the growth of phytoplankton. — *Biochem. Journ.* 27: 1015. 1933.
- Richter, O.: Über Reinkulturen von Diatomeen und die Notwendigkeit der Kieselsäure für *Nitzschia Palea* (Kütz.) W. Sm. — *Verh. Gesell. deutsch. Naturf. u. Ärzte*, 76. Versammlung zu Breslau 1904. II. T., 1. Hälfte: 249. 1904.
- Zur Physiologie der Diatomeen I. — *Sitzb. Akad. Wiss. Wien, mat.-nat. Kl.*, CXV, Abt. 1: 27. 1906.
- Robinson, R. J. & Spoor, H. J.: Photometric determination of silicate in sea water. — *Ind. Eng. Chem. Anal. Ed.*, 8: 455. 1936.
- Rodhe, W.: Environmental requirements of freshwater plankton algae. — *Symbolae Botanicae Upsaliensis* X: 1. 1948.
- Thayer, Lewis A.: Colorimetric determination of silica in the presence of phosphates and iron. — *Ind. Eng. Chem. Anal. Ed.*, 2: 276. 1930.
- Utermöhl, H.: Limnologische Phytoplanktonstudien. — *Arch. Hydrobiol., Suppl.* — Band 5: 1. 1925.
- Wattenberg, H.: Critical review of the methods used for determining nutrient salts and related constituents in salt water. — *Rapports et Procès-Verbaux des Réunions*, 103: 11. 1937.

Osmotic Relations during Cell Expansion

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The general validity of the osmotic relations illustrated by the Höfler — Tamiya diagram (12, 19) has in recent years been questioned by Burström (4, 5, 6) and others. The purpose of the present paper is to examine the cause of the discrepancies and to show how they can be reconciled.

To begin with, it is necessary to give precise definitions of the concepts and terms which we are going to employ.

A. Potential pressures and pressure differences

The osmotic potential (O) of a solution is equal to the maximum osmotic pressure which the solution might develop when transferred to an ideal osmometer (cp. Thoday 20, Levitt 15, 16).

Synonyms: Osmotic pressure, osmotic value (Meyer 18, p. 150). »The osmotic potential of a cell» means the osmotic potential of the vacuolar solution. The term »osmotic pressure» is used in the broad sense recommended by Levitt (15), i.e., indicating any pressure resulting from osmosis.

The osmotic potential difference ($O_1 - O_2$) is the difference between the osmotic potentials of two systems (e.g. cell/external solution). This term has previously been used by Levitt in a different sense (16, see below).

The osmotic equivalent (S) of a system (e.g. plant cell, osmometer, solution) is equal to the osmotic potential of a solution in osmotic equilibrium with the system (Levitt 16).

Synonyms: Suction force, diffusion pressure deficit (Meyer 18, p. 155). We prefer to retain the classical symbol S for this concept, instead of E as proposed by Levitt, for two reasons: The letter E is, in physical literature, preferably employed to denote energy quantities. By using S , the comparison with older literature is facilitated.

The osmotic absorption potential (A) of a plant cell in contact with another system (neighbouring cell, external solution, etc.) is equal to the difference between the osmotic equivalent S_1 of the cell and the osmotic equivalent S_2 of the adjacent system ($A = S_1 - S_2$).

Synonyms: Net suction force, net diffusion pressure deficit (18), osmotic potential difference (Levitt 15, 16). The term »osmotic potential difference» was provisionally used by Levitt to designate the difference between the osmotic equivalents of two systems. This usage is not to be recommended, however, since it is convenient to have a separate term to denote the difference $O_1 - O_2$, i.e., the difference between the osmotic potentials of the systems. Thus it becomes necessary to coin a new term for the difference $S_1 - S_2$, even if this means yet another addition to the already too long list of osmotic terms reviewed by Meyer (18).

Since this difference is a measure of the potential »water absorbing power» of the cell, the term »absorption potential» seems to us the most adequate among the many alternatives which we have taken into consideration. In adding »osmotic» to distinguish the absorption potential in question from active water absorption, solute absorption etc., we have followed a suggestion by Levitt (17).

It should be emphasized that the absorption potential is a relative magnitude, i.e., it has no meaning unless referred to the relation between the cell in question and another, well defined osmotic system. If this other system is distilled water, the absorption potential of the cell is equal to its osmotic equivalent ($S_2 = \text{zero}$, $A = S_1$).

B. Actual pressures

The turgor pressure (P_t) of an osmotic system (e.g. a plant cell) is the actual hydrostatic pressure of the free solution contained in it (cp. Meyer 18, p. 154). We prefer the symbol P_t for the current T in order to indicate that we are dealing with a pressure, and not with an absolute temperature.

The definition given here is in accordance with the original turgor concept as it has been understood by most workers in this field (cp. e.g. 10, 12, 21). We agree with Huber (13), Broyer (3) and Kramer and Currier (14) that it is inadvisable to employ a so well established term in a quite different meaning, as suggested by Burström (l.c.). Burström's turgor is, in fact, a

potential pressure, viz., the osmotic potential difference, $O_1 - O_2$, which is not directly related to the actual state of turgescence of the cell.

We do not see any advantage, either, in the turgor concept introduced by Algeus (1); who defines turgor as the centrifugally directed pressure of the cytoplasmic layer against the cellulose wall. In the first place, we cannot conceive of any practicable method by which this pressure could be experimentally determined. Furthermore, if we consider the intimate connection between cell wall and cytoplasm which obtains in intact cells, a distinction between the elastic tension of the cytoplasmic layer and the elastic tension of the cell wall proper appears as a sort of mathematical artefact.

It deserves to be mentioned that still another turgor definition (and certainly a confusing one) has recently been introduced by Crafts, Currier and Stocking (7, p. 45).

The wall pressure (P_w) of an osmotic system is the total counterpressure exerted by the wall of the container on the free solution contained in it. Thus, in a plant cell both the external pressure of the adjacent tissue and the centripetal pressures resulting from the elastic tension of the cell wall and the elastic tension of the cytoplasmic layer lining the wall, are considered as components of the wall pressure.

As mentioned above, we cannot adopt the deviating definition introduced by Algeus (l.c.) who does not include the cytoplasmic layer as a component of the wall. Even if the corresponding pressure component would certainly be quite negligible, in ordinary cells, as compared with the pressure resulting from the elastic tension of the cell wall proper, this point is theoretically important. (In plasmolyzed cells, the cell wall is no longer part of the osmotic system.)

According to our definitions, turgor pressure and wall pressure are always numerically equal. Where only the numerical magnitude of these pressures is of any consequence and the distinction between outward and inward direction does not matter, we may employ the symbol P to denote either of them ($P = |P_t| = |P_w|$). The fundamental osmotic equation for a plant cell can thus be written:

$$O_1 - O_2 = A + P \quad (1)$$

or, if the cell is immersed in pure water:

$$O = S + P \quad (2)$$

The implications of equation (1) can be illustrated by the extended turgor diagram proposed by Burström (fig. 1):

Here, O_1 is the osmotic potential of the cell, and O_2 the osmotic potential of the external solution. The osmotic potential difference at incipient plas-

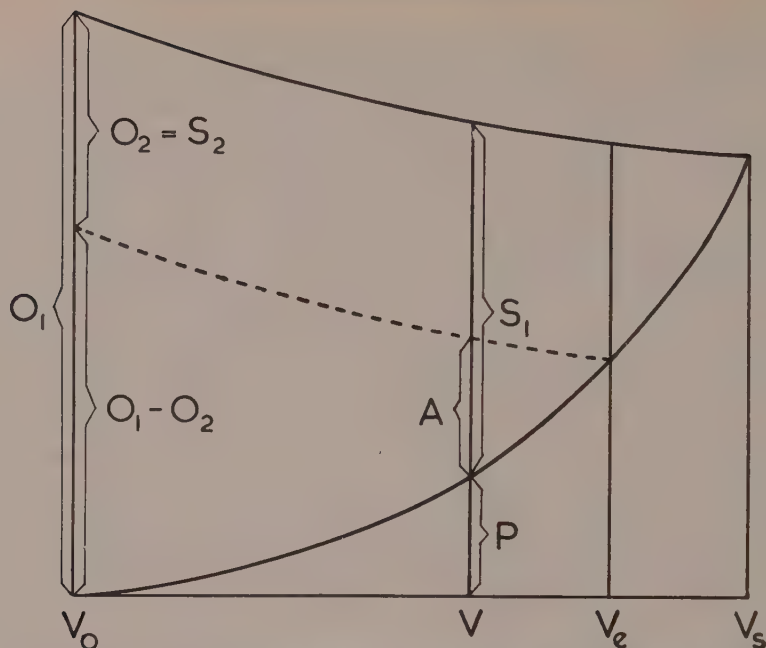


Fig. 1. Burström's extended turgor diagram as interpreted by means of the symbols employed in the present paper.

molysis $(O_1 - O_2)_0$ indicates the maximum turgor pressure which might develop if the cell wall were absolutely rigid. At incipient plasmolysis (volume V_0) the osmotic absorption potential (A) is, therefore, equal to the osmotic potential difference, while turgor (P_t) and wall pressure (P_w) are equal to zero (neglecting the cytoplasmic wall pressure component). During water absorption, the osmotic absorption potential A is gradually realized in the form of an actual pressure, viz., the turgor pressure P_t . The osmotic potential difference is, at the same time, gradually diminished, owing to dilution of the cell sap. (The osmotic potential of the external solution, O_2 , is supposed to remain constant.) Equation (1) is valid at any one time. When osmotic equilibrium is attained, $A = \text{zero}$ and $O_1 - O_2 = P$. At equilibrium, therefore, and only then, does the value of Burström's turgor ($O_1 - O_2$) coincide with the turgor pressure in our definition (P_t).

The part of the turgor curve to the right of the equilibrium point (V_e) in fig. 1 shows the course of the continued water absorption which would take place if the external solution were, at this stage, replaced by pure water, whereby an additional absorption potential equal to S_2 would develop. The final equilibrium would then be attained at water saturation (volume V_s), when $S_1 = \text{zero}$, and $P = O_1$.

According to this interpretation, the only actual pressures included in this diagram, viz., P_t and P_w , are perfectly equilibrated at any one time. The diagram does not, therefore, give any information about the dynamics of the expansion process. No »expanding force», »accelerating force» or »turgor excess» appears in the above relations. Consequently, this diagram may be characterized as an extension of the Höfler — Tamiya diagram only in so far as the osmotic value of the external solution is considered. Both diagrams are valid for static and dynamic states alike, and neither of them contain data pertinent to the dynamics of volume changes.

In the recent discussion it has been maintained that Newton's third law (action=reaction) is only applicable to static states (5, 9, 11). This misapprehension seems to have arisen from a too narrow definition of the concept »wall pressure» and a partial confusion of wall pressure and wall elasticity. It should be emphasized that Newton's law is a general physical principle, valid for both static and dynamic states. According to our definitions, turgor and wall pressure are both real pressures which are always numerically equal, whether the system is at equilibrium or not. But where, then, do we find the »driving force» of cell expansion?

In order to simplify the discussion, let us first consider an ideal osmometer consisting of a perfectly rigid, semipermeable cylinder filled with a sucrose solution. A frictionless piston of mass m and surface area q is fitted tightly in the vertical cylinder and pressed downwards against the solution with a force F by means of a steel spring.

Let the osmotic potential of the solution at the initial stage be O . The total pressure exerted by the piston on the solution is then:

$$P_w = \frac{F + m \cdot g}{q} \quad (3)$$

When the cylinder is now immersed in pure water, the osmometer possesses an absorption potential:

$$A = S = O - P_w$$

which causes water to enter into the cylinder, forcing the piston upwards. This upward movement meets with an additional resistance caused by the inertia of the piston. If a is the acceleration of the piston, then the pressure of the piston against the solution is increased by the amount $\pi = (m \cdot a) : q$. The correct expression for the wall pressure during acceleration is therefore:

$$P_w = \frac{F}{q} + \frac{m \cdot g}{q} + \frac{m \cdot a}{q} \quad (4)$$

The hydrostatic (turgor) pressure of the solution against the piston is, of course, still numerically equal to P_w .

In applying this model experiment to a living cell during osmotic expansion, equation (4) can be written:

$$P_t = P_w = p_e + p_c + \pi \quad (4 a)$$

where p_e is the external pressure on the cell of the adjacent tissue, p_c is the centripetal pressure caused by the elastic tension of the cell wall (including the cytoplasmic layer), and π is the dynamic pressure component caused by the inertia of the wall particles against the movement. This inertia component of the wall pressure has apparently been overlooked by previous authors who have been inclined to invalidate Newton's law in order to construct an accelerating force causing cell expansion.

It is seen from equation (4 a) that the »driving force» of osmotic expansion is not the total turgor pressure, but only the fraction of this pressure which is not balanced by the static components of the wall pressure ($p_e + p_c$). This fraction is numerically equal to the inertia component of the wall pressure (π).

Algeus (1) has tried to derive an equation for »the accelerating force X, which produces the outward movement» by making a distinction between the elastic tension of the cytoplasmic layer and the elastic tension of the cell wall proper. As a consequence of this distinction, he has found it necessary to redefine the concepts turgor and wall pressure in such a manner that they escape experimental determination. In our opinion, this is a quite unnecessary complication. The fundamental equation derived by Algeus for a mechanical model is:

$$(K_1 - e_1 - p) - (K_2 + e_2 - p) = X \quad (5)$$

where X is the »accelerating force» (actually a pressure), and the two p's represent the alleged pressures of the cytoplasmic layer on the cellulose wall and vice versa. Further, in our notation:

$$K_1 = P_t, \quad e_1 + e_2 = p_c, \quad \text{and} \quad K_2 = p_e.$$

Thus, the above equation (5), which can be written in the simplified form:

$$K_1 = (e_1 + e_2) + K_2 + X \quad (5 a)$$

is, in fact, identical with our equation (4 a). Consequently, the »accelerating force» discussed by Algeus is numerically equal to the inertia component π of the wall pressure as defined in the present paper. In this way, the »accelerating force» becomes a definite physical meaning, and the separation of the wall tension into two components is avoided.

A more detailed evaluation of Burström's development now also becomes possible. According to Burström, Newton's third law cannot be applied to the relation between turgor and wall pressure, except at equilibrium. During

osmotic expansion or contraction there is always a difference between them, and it is this pressure difference which represents the »driving force» of the movement. Now, in our notation:

$$\begin{array}{rcl} \text{Burström's turgor} & = & O_1 - O_2 \\ \text{Burström's wall pressure} & = & p_e + p_c \\ \hline \text{i.e., Burström's »driving force»} & = & (O_1 - O_2) - (p_e + p_c) \end{array}$$

Since, according to (1): $O_1 - O_2 = A + P$, and, from (4 a): $p_e + p_c = P - \pi$, we find:

$$\text{Burström's »driving force»} = A + \pi \quad (6)$$

We are unable to find any physical meaning of the sum $A + \pi$ in this equation. However, as we are going to show presently, π is of a much smaller order of magnitude than A (except close to equilibrium when both are approaching zero). Thus, Burström's »driving force» is, in fact, practically identical with the osmotic absorption potential A . A is, however, *par definition*, a *potential* pressure and cannot, as such, act directly as a moving force. It is true, of course, that a positive absorption potential is a necessary condition for water absorption, and that the magnitude of the absorption potential is a measure of the steepness of the free energy gradient which determines the rate of net inward diffusion. Since volume expansion depends on this rate, there is an obvious connection between absorption potential and movement. But this connection is an indirect one. Evidently, the water molecules are unable to exert a pressure on the cell wall from within until they have actually penetrated the wall from the outside and entered into the vacuole. During absorption, the potential pressure A is gradually transformed into an actual pressure, viz., a turgor increase. It is this turgor increase which upsets the static equilibrium and overcomes the inertia of the wall, causing the outward movement. As expansion proceeds, A is gradually reduced towards zero. At the same time, the net invasion of water molecules subsides and the outward movement of the wall is slackened down, while the static components of the wall pressure rise towards a new level where they alone are able to counterbalance the higher turgor.

Though we cannot adopt the definitions proposed by Burström, his demand for some sort of disequilibrium between outward and inward pressures is seen to be fundamentally sound. It should be strongly emphasized, however, that while the turgor pressure during water absorption certainly exceeds the *static* wall pressure, turgor and *total* wall pressure are at every stage perfectly equilibrated.

It is perhaps not always realized that even in living cells substantial turgor variations need not necessarily be accompanied by observable volume changes.

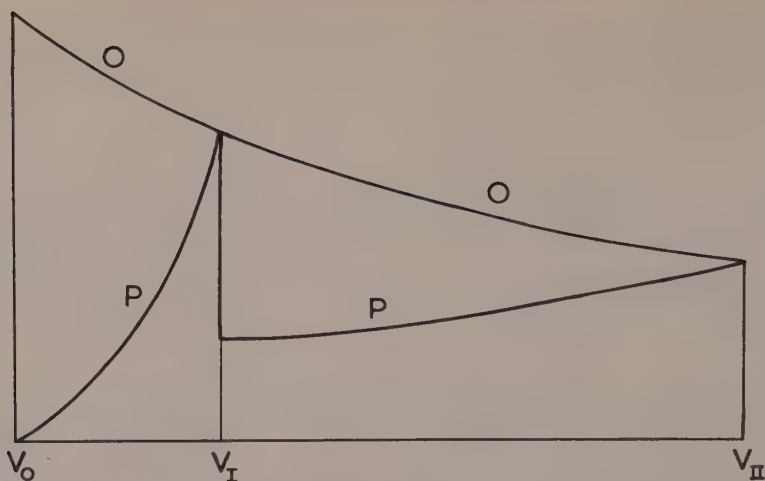


Fig. 2. *Turgor drop during growth in hypothetical model experiment. Explanation in the text.*

Let us consider a water-immersed and fully expanded cell with turgor P at osmotic equilibrium, and study the result of a sudden reduction of the elasticity of the cell wall, such as is often presumed to initiate cell growth (Burström 4, Frey-Wyssling 8).

To take an arbitrary numerical example, we may assume that the static component of the wall pressure of a cell of volume $100 \mu^3$ is reduced from 12 atm. to 4 atm., owing to the reduced wall tension. In order to simplify the treatment, we may further assume that the resulting expansion takes place in two distinct phases: (I) an initial expansion not accompanied by water absorption, and (II) a secondary osmotic expansion. Assuming the compressibility of the cell sap to be the same as that of pure water at 20°C , the volume of the cell is found to increase during phase I from $100 \mu^3$ to $100.04 \mu^3$. This increase certainly falls below the range of microscopic visibility.

At the initial stage $O=P=12$ atm., and $A=S=\text{zero}$. After the completion of phase I, $O=12$ atm. (or strictly 11.995 atm.) and $P=4$ atm. The cell has now developed an osmotic absorption potential: $A=S=O-P=8$ atm., and will begin to absorb water under visible expansion (phase II) until turgor and wall pressure has attained a correspondingly higher level. A schematical illustration of the process is given in Fig. 2.

The sharp distinction between two separate phases which we have made here for technical reasons, do not correspond to the conditions of natural cell growth. In living cells, wall extension and water absorption normally

take place concurrently with an increase of the amount of osmotically active substance in the cell. For this reason, the turgor drop which may actually be observed during the initial growth phase (4) must be much less conspicuous than indicated in Fig. 2 (cp. Abb. 2 by Frey-Wyssling 8).

It follows from these considerations that any visible cell expansion is accompanied by water absorption, and that the volume increase corresponds closely to the volume of water absorbed. Conversely, any appreciable water absorption must cause a corresponding volume increase.

Both water absorption and wall extension requires time. Theoretically, therefore, a measurable turgor excess might develop if the process of wall extension were pretty slow, compared with water absorption. We have no indication whatever that this is so. On the contrary, we have every reason to believe that during water absorption the cell volume, and therefore also the wall tension, is practically instantaneously adjusted to the gradually increasing turgor pressure. It seems reasonable to conclude that the turgor pressure inside a normal cell will scarcely ever exceed the static components of the wall pressure to any measurable extent. In general, therefore, no great error will result if the force of inertia is disregarded in investigations of osmotic relations. Thus, even if a definition of the concept »wall pressure» which includes only static components (p_e and p_c) is theoretically unsound, it may in practice be applied to dynamic states with fairly satisfactory results.

Apparently, even authors who have failed to recognize the physical nature of the »missing link» in the theory of osmosis, viz., the force of inertia, have felt that it may be of minor importance in quantitative respect. Thus Broyer (3) writes: »under conditions of osmotic disequilibrium, . . . a finite but infinitesimal difference obtains between the internal hydrostatic pressure and the wall pressure». A somewhat similar view is probably held by Algeus (1), according to his concluding remarks about infinitesimal and finite differences between inner and outer pressures (i.e., p. 540). Though both these statements are mathematically untenable, they seem to hint at the smallness of the alleged pressure differences.

In order to get an idea of the order of magnitude of the dynamic pressure component π which may be at work in living cells, let us consider a spherical model cell with radius 10 μ and wall thickness 2 μ . The total mass of the wall is then approximately $3 \cdot 10^{-9}$ g. Let us further assume the time required for the cell to attain osmotic equilibrium after immersion in pure water to be 3 min., and the radius of the expanded cell to be 12 μ (i.e. volume ratio 1.728 : 1, average inner surface of wall $q = 1520 \cdot 10^{-8}$ cm²). In order to simplify the calculation, we may assume the dynamic pressure component π

to remain at a constant level during expansion. The acceleration of the wall particles, a , is then:

$$a = \frac{2 \times 2 \times 10^{-4}}{324 \times 10^2} \text{ cm.sek}^{-2} = 1.235 \cdot 10^{-8} \text{ cm.sek}^{-2}$$

The accelerating force, K , is found by multiplication of this value with the total mass, m , of the wall:

$$K = 1.235 \cdot 10^{-8} \cdot 3 \cdot 10^{-9} \text{ dyn} = 3.7 \cdot 10^{-17} \text{ dyn}$$

The pressure component π is then equal to $K : q$, i.e.:

$$\pi = \frac{3.7 \times 10^{-17}}{1520 \times 10^{-8}} \text{ dyn.cm}^{-2} = 2.434 \cdot 10^{-12} \text{ dyn.cm}^{-2} = \text{ca. } 2.4 \cdot 10^{-18} \text{ atm.}$$

In the discussions of osmotic concepts one may sometimes meet with the more or less clearly formulated idea that a cell with a positive osmotic absorption potential is in possession of a sort of stock energy which can be mobilized for osmotic work. This is a misleading view. When a cell is at osmotic equilibrium with its surroundings, the number of water molecules leaving the cell in outward direction per unit time is exactly equal to the number of water molecules entering the cell from the outside. This continuous migration of water molecules in both directions can also be described in terms of energy exchange (2). Outward migration of water molecules means a loss of free energy, inward migration an energy increase. When osmotic equilibrium obtains, an energy balance is established also. If, however, the inward migration for some reason takes the upper hand, a net energy influx must result. The energy required for osmotic expansion therefore originates from the excess specific free energy of the water molecules outside the cell membrane, not from any enigmatic stock energy or preexisting pressure inside the cell. A positive turgor pressure develops gradually, induced by and concurrently with such a net water inflow.

Summary

1. The following osmotic concepts are defined: *Osmotic potential* (O), *osmotic potential difference* ($O_1 - O_2$), *osmotic equivalent* (S), *osmotic absorption potential* ($A = S_1 - S_2$), *turgor pressure* (P_t), and *wall pressure* (P_w). It follows from the definitions given, and from Newton's third law, that the following relations hold good both for static and dynamic states:

$$|P_t| = |P_w| = P, \quad O_1 - O_2 = A + P$$

The implications of these relations at different stages of the expansion process can be illustrated by the extended turgor diagram proposed by Burström. This diagram has no bearing upon the dynamics of cell expansion and contraction.

2. In order to define the »driving force» of osmotic volume changes, it is necessary to distinguish between three separate components of the wall pressure. Two of these components have been generally recognized in previous literature, viz., the centripetal pressure p_e resulting from the elastic tension of the cell wall (including the cytoplasmic layer), and the external pressure p_e exerted on the cell by the adjacent tissue. During dynamic states, the wall pressure is increased by a third component π , resulting from the inertia of the wall particles against acceleration. Hence: $P_w = p_e + p_e + \pi$. The »driving force» of cell expansion originates from the corresponding fraction of the turgor pressure, which is not counter-balanced by the static components of the wall pressure. Consequently, this »driving force» is equal to $\pi \cdot q$, where q is the inner surface area of the wall.
3. It is shown by an arbitrary numerical example that the dynamic component of the wall pressure (π) is likely to be of an extremely small order of magnitude, as compared with the static components ($p_e + p_e$). In ordinary plasmolysis experiments, therefore, the dynamic pressure component can safely be neglected.
4. The alterations of the usual definitions of turgor and wall pressure recently proposed by Burström (4, 5, 6) and Algeus (1) are discussed and rejected.

References

1. Algeus, S.: Views on turgor pressure and wall pressure. — *Physiol. Plant.* 4: 535. 1951.
2. Broyer, T. C.: The movement of materials into plants. Part 1. Osmosis and the movement of water into plants. — *Bot. Rev.* 13: 1. 1947.
3. — On the theoretical interpretation of turgor pressure. *Plant Physiol.* 25: 135. 1950.
4. Burström, H.: Die osmotischen Verhältnisse während des Streckungswachstums der Wurzel. — *Lantbrukshögsk. Ann.* 10: 1. 1942.
5. — A theoretical interpretation of the turgor pressure. — *Physiol. Plant.* 1: 57. 1948.
6. — Mechanisms of cell elongation. — *Plant growth substances* (ed. F. Skoog): 43. Wisconsin 1951.
7. Crafts, A. S., H. B. Currier and C. R. Stocking: *Water in the physiology of plants.* Waltham 1949.
8. Frey-Wyssling, A.: Über die Dehnungsarbeit beim Streckungswachstum pflanzlicher Zellen. — *Vierteljahrsschr. Naturforsch. Ges. Zürich* 93: 24. 1948.
9. — Physiology of cell wall growth. — *Ann. Rev. Plant Physiol.* 1: 169. 1950.
10. Haines, F. M.: The relation between cell dimensions, osmotic pressure and turgor pressure. — *Ann. Bot.* 14: 385. 1950.
11. — The dynamics of cell expansion by turgor. — *Ann. Bot.* 15: 219. 1951.

12. Höfler, K.: Ein Schema für die osmotische Leistung der Pflanzenzelle. — Ber. Dtsch. Bot. Ges. 38: 288. 1920.
13. Huber, B.: Wasserumsatz und Stoffbewegungen. — Fortschr. Bot. 12: 185. 1949.
14. Kramer, P. J., and H. B. Currier: Water relations of plant cells and tissues. — Ann. Rev. Plant Physiol. 1: 265. 1950.
15. Levitt, J.: Toward a clearer concept of osmotic quantities in plant cells. — Science 113: 228. 1951.
16. — The osmotic equivalent and osmotic potential difference of plant cells. — Physiol. Plant. 4: 446. 1951.
17. — Personal communication. 1952.
18. Meyer, B.: A critical evaluation of the terminology of diffusion phenomena. — Plant Physiol. 20: 142. 1945.
19. Tamiya, H.: Zur Theorie der Turgordehnung und über den funktionellen Zusammenhang zwischen den einzelnen osmotischen Zustandsgrößen. — Cytologia 8: 542. 1937.
20. Thoday D.: On the water relations of plant cells. — Ann. Bot. 14: 1. 1950.
21. Ursprung, A., und G. Blum: Dürfen wir die Ausdrücke osmotischer Wert, osmotischer Druck, Turgordruck, Saugkraft synonym gebrauchen? — Biol. Zbl. 40: 193. 1920.

On the Specificity and Products of Action of Xylanase from *Chaetomium globosum* Kunze

By

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The first stage in the biological decomposition of xylan is the conversion into soluble sugars. This conversion is brought about by enzymes known as hemicellulases or cytases. The names xylanase, mannanase, arabanase etc. are often in the literature applied to enzymes hydrolyzing the different hemicelluloses, but the specificity of these enzymes has been studied very little; most investigations on enzymatic degradation of xylan and other hemicelluloses have been carried out with crude enzyme extracts prepared from snails, malt, or commercial enzyme preparations as taka diastase or luizym.

The literature on the biochemistry, the methods of extractions and the fractionation of hemicelluloses has been reviewed by Norman (10) and Pigman and Goepp (15), and that on hemicellulases by Weidenhagen (22) and Pigman (16).

In this paper experiments are reported on enzymatic hydrolysis of wheat straw xylan. The problem of the specificity of the xylanase has been studied by investigating the formation of the enzyme on various substrates, utilizing the fact that the xylanase of *Chaetomium* is adaptive. Reaction products of xylanase have been studied by the method of paper partition chromatography.

Methods and materials

Methods of analysis

Reducing sugars were determined by the micromethod of Stiles *et al.* (19), *Furfural* by distillation with 12 per cent hydrochloric acid after Kullgren and Tydén,

the evolved furfural being measured by bromine oxidation (Bengtsson, 2), *Lignin* by the thioglycollic acid method of Holmgren as used by Bengtsson (2).

Substrates

Xylan. (References see Weihe and Phillips, 23). Mature wheat straw was cut into one-cm pieces and extracted successively at 80—90° for 6 hours with (a) water, (b) 0.5 per cent ammonium oxalate, (c) water; then 96 per cent ethanol at room temperature for 16—20 hours, and extraction for 45 min. in the autoclave in flowing steam with 1-N sodium hydroxide after standing overnight at room temperature. After neutralization with acetic acid the xylan was precipitated with 1.5 vol 96 per cent ethanol. About 20 per cent crude xylan with 6—8 per cent water was recovered. Analysis of a xylan preparation is shown in Table 1.

Acid hydrolysis of the xylan. A sample of xylan was boiled for 6 hours with 1-N sulphuric acid under reflux. After centrifugation the clear solution was neutralized with barium hydroxide. The barium precipitate was washed with hot water and the combined hydrolyzate and washing was concentrated on a water bath. Barium uronate was precipitated with excess of ethanol. The ethanolic sugar solution freed from the barium uronate was again concentrated and the sugars taken up in a little water. By chromatography of the sugar solution xylose, arabinose and glucose were demonstrated; the main constituent was xylose judging from the intensity of the spots. The barium uronate was reprecipitated and dried with ethanol and ether; 25 mg and 1 ml 1-N sulphuric acid were sealed in a glass ampoule and heated for 8 hours in boiling water. After adjustment with barium hydroxide to pH 3.6, filtration, and concentration, the presence of xylose and a uronic acid was demonstrated chromatographically. These findings are in accordance with those of Bishop and Adams (4) and Adams and Castagne (1) who on acid hydrolysis of wheat straw hemicelluloses extracted from holocellulose demonstrated xylose and arabinose and a small constant amount of glucose, and sometimes galactose in various fractions, besides a barium uronate complex which on hydrolysis yielded xylose and a uronic acid tentatively identified as galacturonic acid.

Preparation of colloidal xylan solution. (Voss and Butter, 20). Xylan powder was brought in solution in a mortar with 5—6 ml 1-N sodium hydroxide per gm of xylan, diluted with water to 4—5 per cent xylan, heated to 40—50° for half an hour on a water bath. After centrifugation from insoluble particles the clear solution was dialyzed in collodion sacks to pH about 6.5 (2—3 days). The solution was then concentrated in vacuo (50°) and furfural determined in 5 or 10 ml and the concentration adjusted to 5.7 mg/ml furfural (10 mg/ml xylose after Kröber's tables, 8). The solution was stored in a bottle with excess of toluene and sterility checked now and then. A very slight deposit appeared after standing for months, but there was no difference in rate of enzymatic hydrolysis by new and older preparations. Reducing power: 0.02—0.03 mg/ml glucose, pH 6.5. Analysis of xylan precipitated with 5 vol. ethanol from a colloidal solution is shown in Table 1.

Mannan. Tuber salep powder was extracted repeatedly with cold water, and after centrifugation the combined extracts were concentrated on a water bath and the mannan precipitated with 3 vol. ethanol. After washing with ethanol and ether the precipitate was dried in vacuo. It gave a brown colour with iodine and contained 7.6 per cent water, 1.7 per cent ash and 0.5 per cent furfural. Reducing sugar after hydrolysis for 6 hours with 1-N sulphuric acid was 88.3 per cent of ash-free dry

Table 1. Analysis of xylan (per cent of ash-free dry matter).

Compounds	Crude xylan	Xylan precipitated from the colloidal solution
Furfural evolved	53.5	53.7
Xylose, from furfural after Kröber (8)	94.2	94.7
Xylan » » » »	83.0	83.5
Reducing sugar after acid hydrolysis	81.0	81.2
Residue after acid hydrolysis.....	7.4	4.8
Lignothioglycollic acid	3.9	
Residue after dissolving in NaOH	4.0	
Ash (per cent of dry matter)	7.4	1.9

matter. A solution containing 1 per cent reducing sugar (after hydrolysis) was used in the experiments. Reducing power: 0.078 mg/ml (as glucose).

Cellulose-dextrin was prepared from cotton after Fuller and Norman (5). The water-insoluble cellulose-dextrin used in the experiments was stored as a suspension with 10 mg dry matter per ml, kept sterile with toluene. Reducing power: 0.166 mg/ml glucose.

Starch. A 1 per cent solution of soluble starch was used.

»*Galactan*» was extracted from *Chondrus crispus* (Irish moss) with hot water and after concentration on a water bath precipitated with 3 vol. ethanol.

Enzyme preparations

The organism. *Chaetomium globosum* Kunze was isolated from soil and determined by Prof. N. Fabritius Buchwald, The Royal Veterinary and Agricultural College, Copenhagen. The basal medium was a solution of the following inorganic salts in tap water (gm/l): NH_4NO_3 1.0; K_2HPO_4 0.25; KH_2PO_4 0.25; MgSO_4 0.2; NaCl 0.2; FeCl_3 0.02; carbon source 10 gm/l. Inoculation was taken from cultures on filter paper strips in the basal mineral solution. Fig. 1 shows decomposition of wheat straw by *Chaetomium globosum*. 500 gm one-cm pieces of mature wheat straw and 50 ml of the basal mineral solution in 300 ml Erlenmeyer flasks. 30 min. in the autoclave at 120° . Inoculation with a spore suspension. Incubated at 25° together with uninoculated controls. Analysis according to Waksman and Stevens (21). No lignin decomposed.

Dry mycelium powder was prepared according to Bernhauer and Knobloch (3). *Chaetomium* was cultivated on the basal medium with the different substrates as carbon source in one-liter Erlenmeyer flasks at 25° . (Xylan was dissolved in 1-N sodium hydroxide and the mixture neutralized with acetic acid). As soon as the surface was covered (xylan 2 to 3 days, the other substrates 3 to 4 days), the mycelium was harvested, thoroughly washed with cold water, and kept overnight with acetone; it was then ground in a mortar under acetone which was filtered off, again washed with acetone and ether, and dried in vacuo. When kept in tightly closed bottles in a cool place in the dark the preparations remained stable for a long time. Very active preparations prepared after growth on xylan lost 15—25 per cent of their activity within the first 2—3 weeks and then became stable. Pre-

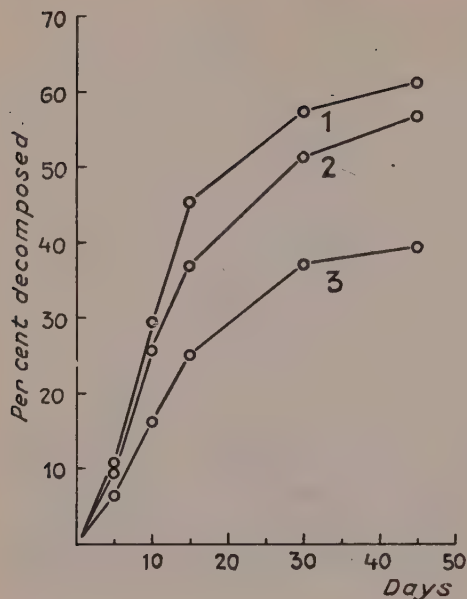


Fig. 1. *Decomposition of wheat straw by Chaetomium globosum*. 1. Cellulose, 2. Hemicellulose, 3. Total dry matter.

parations dried from water in vacuo had the same activity as preparations dried by means of acetone.

Cell-free enzyme solutions were obtained by extracting xylan-grown, dry mycelium powder with water and a little toluene for 16–20 hours. After centrifugation the solution was filtered through a Jena glass filter 11 G 5 on 3. About 65 per cent of the activity was removed by two extractions; the extracted and redried mycelium had about 60 per cent of the activity of the untreated dry mycelium.

Determination of enzyme activity

The activity was measured by the amount of reducing sugars produced by dry mycelium in a 0.5 per cent solution of the substrate in phosphate buffer of pH 6.5. 15 mg dry mycelium + 5 ml $1/15$ -M phosphate buffer + 5 ml 1 per cent substrate solution + 2 ml toluene in 50 ml cork-stoppered Erlenmeyer flasks, shaken vigorously and placed in the incubator at 37° C. Initial reducing sugar for the different substrates + enzyme preparations 0.1–0.15 mg glucose per ml. At intervals 1 ml was removed for determination of reducing sugar directly in the substrate. It was ascertained that none of the compounds added had any influence on the determination of glucose. All values for reducing sugars are expressed as glucose.

Analysis of hydrolysates by paper chromatography

Descending flow was used in an all-glass jar at 25°. Solvent: butanol + glacial acetic acid + water (4 : 1 : 5). Developer: aniline hydrogen phthalate (Partridge, 12, 13). Paper: Schleicher & Schüll 2043 b.

The phosphate buffer was replaced by distilled water where hydrolysates were

to be analysed. The undecomposed xylan was precipitated with 2 vol ethanol and removed by centrifugation, the solution was evaporated to dryness on a water bath, and 2 ml 30 per cent ethanol was added to precipitate the rest of the undecomposed xylan. After centrifugation in 2.5 ml tubes the completely clear solution was condensed to approximately 1 per cent reducing sugar in small glass cups on a sand bath at 50 to 60°, and 2 to 4 μ l were spotted on the paper by means of a capillary pipette. Pure sugar solutions were spotted on the same sheet for identification.

Experimental

Factors affecting the xylanase activity

Fig. 2 shows the effect of *pH* upon the activity of xylanase. The experiment was carried out by adding 5 ml $1/15$ -*M* phosphate buffer solution of the desired *pH*. The optimum *pH* is seen to be 6.5. Mannanase and cellulase of *Chaetomium globosum* had the same *pH* optimum.

Fig. 3 shows the effect of *temperature* upon xylanase activity. Optimum at these conditions is seen to be 37° C. The activity at 42° is decreasing and the activity at 30° is increasing compared to the activity at 37° during the experiment, probably because the destruction of the enzyme increases with increasing temperature.

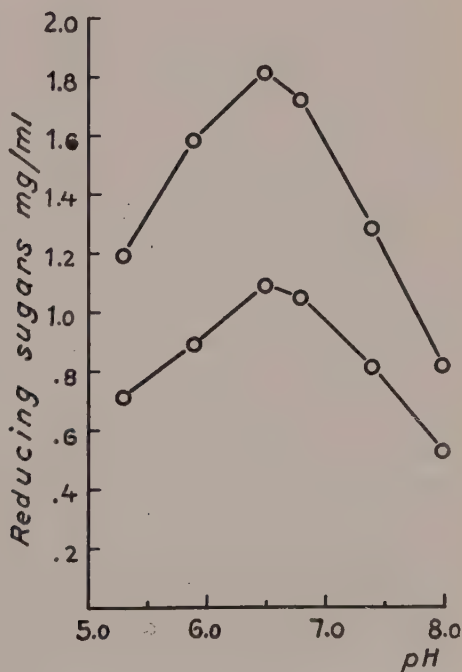


Fig. 2. Influence of *pH* on enzymatic hydrolysis of xylan in 72-hours experiments. Upper curve: 0.5 per cent xylan; lower curve: 0.25 per cent xylan.

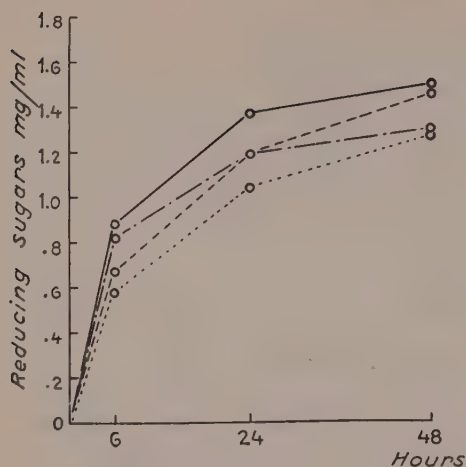


Fig. 3. Influence of temperature on enzymatic hydrolysis of xylan. —•— 42°; ——— 37°; - - - 30°; ···· 25° C.

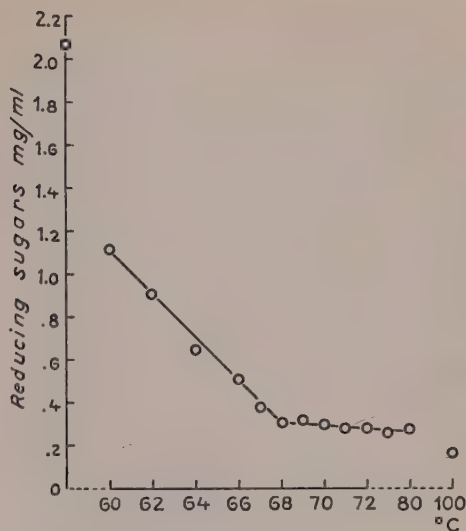
Experiments with increasing amounts of enzyme material have shown that below 15 mg dry mycelium (xylan-grown) the amount of sugar produced is approximately proportional to the amount of dry mycelium, but with greater amounts of dry mycelium the activity per mg dry mycelium is decreasing.

In Fig. 2 the two curves indicate the activity at different pH-values for two different xylan concentrations: The upper curve 0.5 per cent xylan, the lower curve 0.25 per cent xylan. By doubling the substrate concentration the activity is increased 66 per cent at pH 6.5, 58 per cent at pH 5.3, and 55 per cent at pH 8.0.

Fractions and source of xylan. Samples of xylan »A» and »B» were prepared by successive addition of acetic acid and ethanol to the alkaline extract. A sample of wood xylan was prepared from sawdust (mixture of various sorts of wood). Xylan »A» was less readily soluble than xylan »B». Ash content: »A» 1.7 per cent; »B» 7.2 per cent. No significant difference in rate of enzymatic hydrolysis was observed between xylans »A», »B», »A» + »B» (no fractionation), and wood xylan.

Inactivation by heat. Fig. 4 shows the decrease in xylanase activity caused by heating of the enzyme material. 15 mg dry mycelium grown on xylan + 5 ml phosphate buffer of pH 6.5 was heated for 10 min. on a water bath. After cooling, the xylan and toluene were added and the flasks placed in the incubator for 72 hours. The activity decreases very rapidly by heating to 60°. Heating at 60° to 68° diminish the activity gradually, but heating at 68° to 80° affects the residual activity very slightly, and even after heating to 100° there is some activity left. The mycelial matter probably protects

Fig. 4. Influence of heating the xylanase preparation. 15 mg xylan-grown dry mycelium suspended in 5 ml $1/15$ -M phosphate buffer (pH 6.5) was heated for 10 min. on a water bath, after cooling addition of xylan and toluene. 72 hours in the incubator. Blank subtracted.



the enzyme, because the activity in an enzyme extract is destroyed by heating to 68° for 10 min.

Influence of various salts on the xylanase activity. For these experiments an acetate buffer of pH 6.2 in a final concentration of $1/30$ -M was used. The rate of hydrolysis was the same in an acetate buffer and a phosphate buffer of the same pH. The salts were added from a solution adjusted to pH 6.2 with sodium hydroxide. The activity was determined after 24 hours. Correction for reducing power proper of the salts.

The activity was completely suppressed by $1/100$ -M concentration of lead nitrate, mercuric chloride and silver nitrate, about 50 per cent suppressed by copper sulphate and about 30 per cent suppressed by cobaltous chloride.

Weak inhibition (10—15 per cent) was caused by sodium iodo-acetate, sodium azide and sodium fluoride in $1/100$ -M concentration.

No inhibition was found with sodium cyanide, sodium molybdate and urethan in concentrations of $1/100$ -M.

Specificity of the xylanase

Figs. 5 to 8 show the result of experiments on specificity. The organism was grown on the basal medium with xylan, mannan, cellulose-dextrin, starch, xylose and glucose, respectively, as the sole carbon source. The mycelium was harvested, dry mycelium prepared, and the activity against the same polysaccharides determined. Reducing sugar was produced by autolysis of the dry mycelium itself. Blank experiments in which the sub-

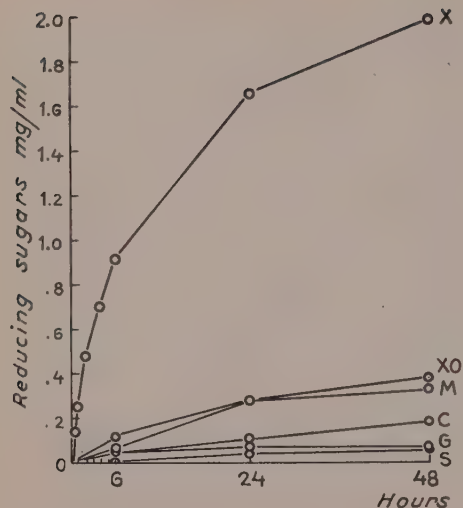


Fig. 5. Hydrolysis of xylan by dry mycelium of *Chaetomium globosum* grown on: X, xylan; M, mannan; C, cellulose-dextrin; S, starch; G, glucose; XO, xylose.

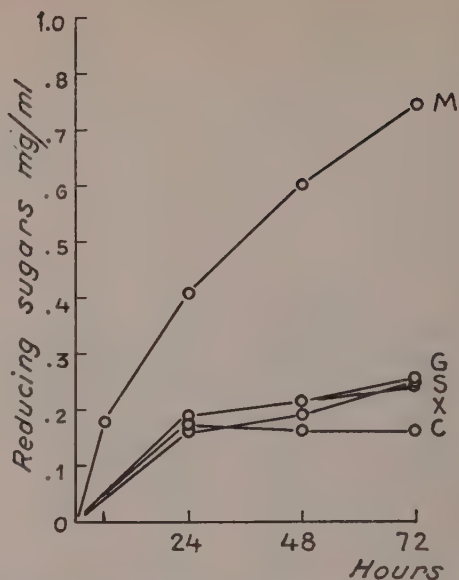


Fig. 6. Hydrolysis of mannan; for explanation see legend of Fig. 5.

strates were replaced by water were therefore carried out with the mycelium preparations, and reducing sugar determined at the same time intervals as in the experiments with substrate; the values (0.15 to 0.29 mg/ml/24 hours for the different preparations) were subtracted from those obtained in the experiments with substrate.

Fig. 5 shows that the amount of xylanase in xylan-grown mycelium is many times the amount in mycelium grown on the other carbon sources; this indicates that xylanase in *Chaetomium globosum* is an adaptive enzyme different at least from mannanase, cellulase and diastase. It is seen that the production of xylanase is not increased by growth on xylose. Adaptive enzymes may be produced by growth on the product of enzyme activity; Phaff (14) who studied the production of exocellular pectic enzymes by *Penicillium chrysogenum* on various substrates, thus found that these enzymes were adaptive, and besides on pectin and some other related substrates, they were also produced by growth on d-galacturonic acid.

Fig. 6 shows that mannanase also is an adaptive enzyme, different at least from xylanase, cellulase and diastase. Fig. 7 shows the cellulase activity of the different preparations. It is seen that the activity of mycelium grown on xylan, cellulose-dextrin and mannan is of the same rate. Fig. 8 shows

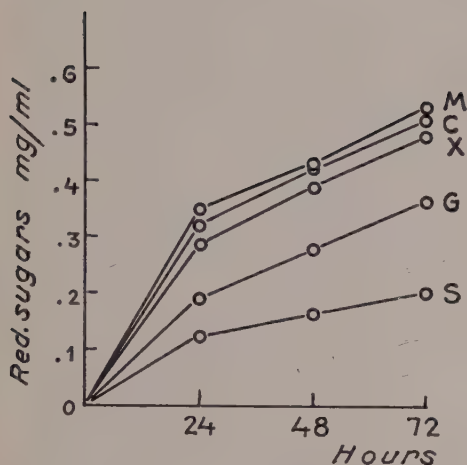


Fig. 7. Hydrolysis of cellulose-dextrin; for explanation see legend of Fig. 5.

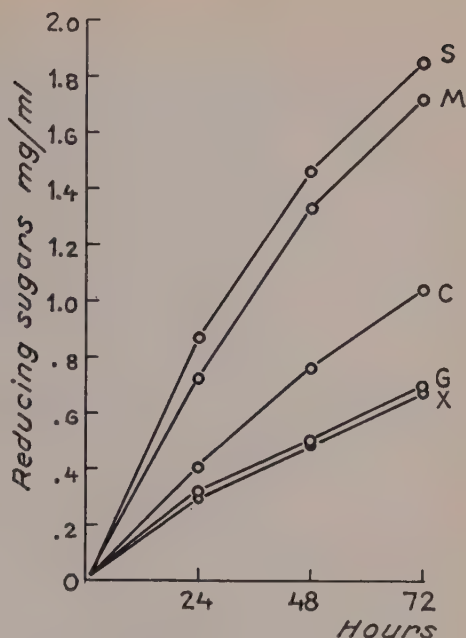


Fig. 8. Hydrolysis of soluble starch; for explanation see legend of Fig. 5.

that the diastase activity in starch and mannan-grown mycelium is of the same rate. The mannan preparation was coloured brown with iodine, indicating that no or little starch was present. Thus it is questionable whether the increased diastase activity in mannan-grown mycelium may be explained as being due to the presence of starch in the substrate.

Chaetomium was also grown on »galactan» and inulin, but the growth was so poor that it appears doubtful if the fungus possesses enzymes active against these compounds. A small amount of mycelium was, however, collected but the activity against xylan was of the same rate as that of glucose-grown mycelium.

The findings are in accordance with those of Grassman, Stadler and Bender (6) who studied the specificity of cellulose- and hemicellulose-splitting enzymes in extracts of *Aspergillus oryzae* (Luizym). These authors were able to remove the xylanase activity from the extracts by adsorption on charcoal, but the cellulase activity was left, indicating that xylanase and cellulase are different enzymes. A weak activity against mannan and inulin could be removed by dialyzing the crude enzyme extract.

Products of hydrolysis

On prolonged action xylanase in dry mycelium was able to hydrolyze 55 to 60 per cent of the xylan, judging from the amount of reducing sugar produced. Paper-chromatographic analysis of the hydrolysates showed that xylose was the principal product, but arabinose was also present; the amount of arabinose in proportion to xylose appeared to be greater if an extracted and redried mycelium was used (Fig. 9 III). Fig. 9 IV shows no arabinose spot, the concentration of arabinose in proportion to xylose is so slight that the arabinose spot is only detectable if the solution is rather concentrated and the run extremely long. Glucose spots also appeared on the paper, but compounds in the dry mycelium itself were autolyzed under the formation of glucose exclusively. Because of this it was not possible to decide if glucose was split off from the xylan by the enzyme. Even in aqueous extracts of dry mycelium after only one hour of extraction glucose was present. Comparison of the intensity of the glucose spots from experiments with and without xylan suggested that if glucose was split off from the xylan, it was only in slight amounts; extracted and redried mycelium in which most of the glucose-yielding compound had been hydrolyzed and glucose washed away yielded very faint glucose spots (Fig. 9 III).

Spots indicating uronic acid were never observed on the paper. The following experiment was therefore performed: 100 mg xylan-grown, dry mycelium was added to 50 ml 1 per cent xylan solution and every third day another 100 mg was added, until a total of 400 mg. After 12 days no increase in reducing sugar was observed, and about 50 per cent of the xylan was hydrolyzed. The undecomposed xylan was precipitated as described above. The pH of the clear hydrolysate was about 3.6. Barium hydroxide was added to pH 7.8, then 5 vol. ethanol were added, and a precipitate appeared. The barium salt was removed by centrifugation, then dissolved in water, filtered and reprecipitated twice. About 60 mg of precipitate was recovered. The combined ethanolic solutions freed from the barium salt were evaporated to dryness on a water bath, and the sugar taken up in a little water.

The chromatographic analysis of the fractions demonstrated that the hydrolysate not treated with barium contained xylose, a little arabinose, glucose and a component giving a spot not moving from the starting line. (Glucose is formed by autolysis of the dry mycelium).

The hydrolysate from which the barium salt was removed showed xylose, arabinose and glucose, but no spot on the starting line.

Aqueous solutions of the barium salt with and without removal of Ba^{++} by addition of sulphuric acid showed only a non-moving spot on the starting line.

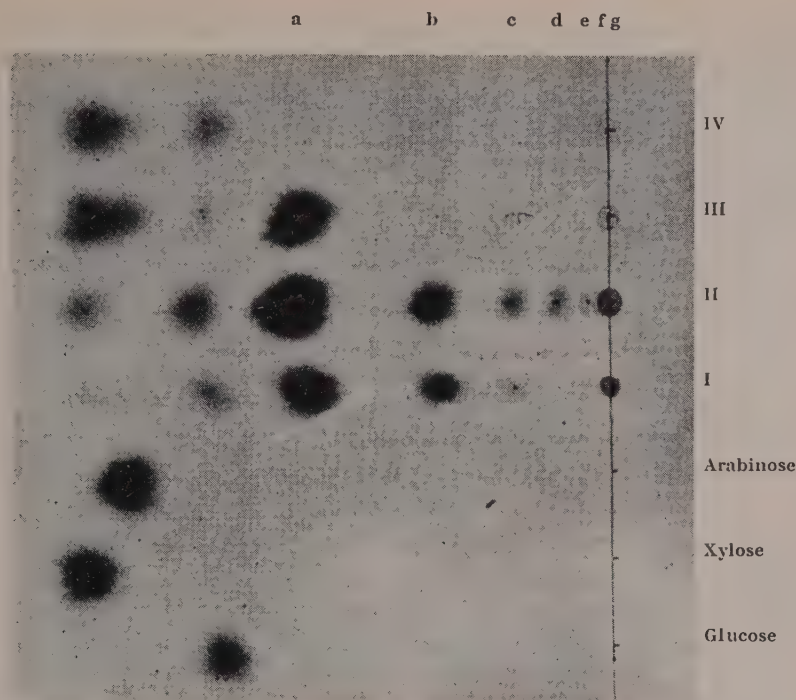


Fig. 9. *Products of enzymatic hydrolysis of xylan.* I. An aqueous cellfree extract of xylan-grown dry mycelium stored for about one month at 12 to 15°. II. A fresh aqueous cell-free extract. III. Extracted and redried mycelium. IV. Untreated dry mycelium; after hydrolysis for 72 hours a fresh amount of dry mycelium was added. *a* to *g* indicate unknown intermediates.

The barium salt was subjected to acid hydrolysis in the following way: 25 mg and 1 ml 1-*N* sulphuric acid were sealed in a glass ampoule, heated in boiling water for 6 hours, barium hydroxide was then added to pH 3.6, the precipitate was removed by centrifugation, and the supernatant solution concentrated to about 0.2 ml. The hydrolysate showed xylose, arabinose, glucose and a uronic acid (no spot on the starting line). Judging from the intensity of the spots the three first were present in approximately equal concentrations, the uronic acid in a lower concentration.

When the barium salt was dissolved in water for the first time a non-water-soluble deposite appeared. It was filtered off and hydrolyzed as described above. The hydrolysate showed xylose, arabinose and glucose, no uronic acid spot was observed.

This experiment shows that a water-soluble, enzyme-resistant polysaccharide consisting of xylose, arabinose, glucose and uronic acid units is

produced from the xylan; this compound does not move on the paper but is coloured by the aniline phthalate developer.

O'Dwyer (11) studied the hydrolysis of hemicellulose A from English oak by means of taka diastase. Prolonged hydrolysis resulted in almost complete fractionation into a water-soluble, enzyme-resistant polysaccharide and xylose (in the proportion of 2 : 3 by weight). Acid hydrolysis of the water-soluble polysaccharide yielded xylose and a uronic acid, in amounts that agreed with a theoretical molecule consisting of one hexuronic acid unit and six xylose units.

Intermediates in the hydrolysis

If a weak enzyme preparation (especially an aqueous extract of dry mycelium) was used, seven unknown spots moving slower than the pentoses and the hexoses were observed on the chromatogram (Fig. 9 II). These spots have the same colour as the xylose and arabinose spots with the aniline hydrogen phthalate developer, and they are not removed by the precipitation of the water-soluble polysaccharide as described above. The soluble polysaccharide is not precipitated from the hydrolysates used in Fig. 9, so that the non-moving spots are seen on the starting line; other experiments where the soluble polysaccharide was removed showed also a spot on the starting line when a proper enzyme preparation was used, indicating a compound that does not move on the paper but is different from the soluble polysaccharide.

The spots are designated as *a* to *g* on Fig. 9. The first, *g*, does not move from the starting line, the next, *f*, is not visible on the foto but was detectable on the chromatogram. The center of the spot is situated approximately on the edge of the non-moving spot *g*.

The *R_f* values of the compounds represented by the spots *a* to *f* for the butanol-acetic acid-water system were: *a*, 0.208; *b*, 0.119; *c*, 0.068; *d*, 0.036; *e*, 0.015; *f*, 0.007; (xylose, 0.339).

The spots *g* to *b* disappear from the chromatogram if a more active preparation is used (Fig. 9 III). The spot *a* disappears on prolonged hydrolysis with a fresh amount of dry mycelium, and only spots indicating xylose, arabinose (glucose) and the soluble polysaccharide are seen.

Samples taken out at intervals during hydrolysis showed on chromatographic analysis that one hour after the start of the experiment a xylose spot was visible on the paper (4 ml samples); after 24 hours of hydrolysis spot *a* also appeared on the paper and disappeared only after addition of a fresh amount of dry mycelium. This phenomenon suggested that two components were active in the hydrolysis of xylan to xylose, the first leading to compound *a*, the second hydrolysing *a* to xylose, and that the rate of destruction was

greater for the second than for the first, resulting in an accumulation of compound α . Experiments showed that if dry mycelium was heated in aqueous suspension for 10 min. at temperatures from 60° to 68°, the xylose spot on the chromatogram grew smaller and smaller and reached a minimum at 68° where only a shade of xylose was visible, whereas the slower moving spots (especially α) remained at the same intensity. This relation between the spots was the same even on heating to 80°. If a cell-free extract of dry mycelium was stored for about a month at 12 to 15°, no xylose spot appeared on the chromatogram, and only the slower moving spots, especially α , were observed. The enzyme preparation had thus lost all activity towards compound α ; some of the activity leading to the formation of this compound still remained.

Discussion

The slower moving spots which appeared on the paper when a weak enzyme preparation was used, and which gradually disappeared on the use of more active preparations may be explained as intermediates in the hydrolysis leading to the formation of xylose (and arabinose). Jermyn and Tomkins (7) who studied the course of the enzymatic hydrolysis of pectin by means of paper-chromatography observed at the beginning of the hydrolysis a number of spots moving slower than galacturonic acid which is the end product of the hydrolysis. As the hydrolysis proceeded the slower moving spots waxed in size, then waned and disappeared, and at the end of the hydrolysis only the galacturonic acid spot was seen. They discuss the different ways in which a polysaccharide chain may be split by enzymes: 1) by removing single sugar units from the end of the chain. 2) by removing groups of units from the end of the chain, followed by hydrolysis of the oligosaccharides by a second enzyme, or (3) by random scission of the chain, leading eventually to single sugar units. They interpret their observations as indicating that polygalacturonase acts by random scission of the glycosidic linkages of the polygalacturonic chain, resulting in molecular fragments with decreasing number of galacturonic units; the slower moving spots represent such molecular fragments with 5, 4, 3, or 2 units. This explanation is supported by the regularity of the R_f values of galacturonic acid and of the compounds represented by the slower moving spots. Only fragments with 5 units or less are able to move on the paper.

This interpretation may also be applied to the slower moving spots observed in this investigation (Fig. 9). The regularly decreasing R_f values suggest compounds of regularly increasing molecular complexity. The red colour of the spots indicate xylose or arabinose units. Uronic acid is not present because

the compounds represented by the spots are not precipitated by barium, and no uronic acid spot appears on complete hydrolysis (Fig. 9 IV). The spots *a* to *g* may thus represent oligosaccharides or molecular fragments consisting of 2 to 8 xylose (or arabinose) units. That spot *a* represents a compound consisting of two xylose molecules is supported by the fact that the ratio $R_f \text{ glucose}/R_f \text{ cellobiose}$ is the same as $R_f \text{ xylose}/R_f \text{ comp. } a$.

Experiments with heating of mycelium and storing of an enzyme extract resulted in no or a very slight production of xylose and an accumulation of comp. *a*; this fact suggests that two components are present in the enzyme hydrolysing xylan, the first leading to comp. *a*, and the second hydrolysing *a* to xylose; the second appears to be more easily inactivated than the first. This interpretation would be in good agreement with the enzymatic hydrolysis of cellulose as shown by Pringsheim (17, 18) and recently confirmed by Levinson, Mandels and Reese (9) by means of the paper chromatography technique. The cellulose chain is hydrolyzed by cellulase under the formation of cellobiose, and this again is hydrolyzed to glucose by cellobiase (β -glucosidase). In experiments with thermophilic cellulose-decomposing bacteria, carried out at 67°, the cellobiase was inactivated whereas some cellulase activity was still present, resulting in an accumulation of cellobiose in the medium (Pringsheim, 17). The storing of an enzyme extract also inactivated the cellobiase whereas the cellulase remained active and an accumulation of cellobiose took place (Pringsheim and Kusenack, 18).

If the above explanation, i.e., that two components are present in xylanase, is correct, comp. *a* may be analogous to cellobiose and termed »xylobiose».

Summary

1. Experiments with enzyme preparations produced from *Chaetomium globosum* grown on various polysaccharides showed that xylanase is an adaptive specific enzyme, which is only produced in the presence of xylan in the medium.

2. Paper-chromatographic tests proved that the principal products of hydrolysis are xylose, besides a small amount of arabinose and a water-soluble, enzyme-resistant polysaccharide. This polysaccharide does not move on the paper chromatogram, and yielded on acid hydrolysis xylose, arabinose, and glucose in approximately equal amounts together with a comparatively slight amount of a uronic acid.

3. When proper xylanase preparations are used a series of spots moving slower than the pentoses and the hexoses and with regularly decreasing R_f values appear on the chromatogram. These spots are interpreted to represent

molecular fragments with increasing number of xylose units, resulting from random scission of the glycosidic linkages of the polyxyloside chain. The fastest moving of these compounds may consist of two xylose units and be an analogue of cellobiose.

4. Heating or storing of xylanase preparations resulted in almost complete removal of the power to produce xylose, whereas slower moving spots representing intermediate products of hydrolysis still appeared on the chromatogram, suggesting that two components are present in the xylanase.

My thanks are due to Professor N. Fabritius Buchwald for the determination of the *Chaetomium* sp., and to the chief of the bacteriological department Dr. H. L. Jensen for his interest in this investigation.

References

1. Adams, G. A. & Castagne, A. E.: Hemicelluloses of wheat straw. — *Can. J. Chem.* 29:109. 1951.
2. Bengtsson, N.: Decomposition of cellulose, pentosans and lignin in soil. — *Ann. Agric. Coll. Sweden* 3:1. 1936.
3. Bernhauer, K. & Knobloch, H.: Gewinnung wirksamer Zellpräparate aus Schimmelpilze. — *Bamann & Myrbäck: Methoden d. Fermentforschung* p. 1303. 1941.
4. Bishop, C. T. & Adams, G. A.: Use of liquid ammonia in the fractionation of wheat straw holocellulose. — *Can. J. Res. B* 28:753. 1950.
5. Fuller, W. H. & Norman, A. G.: A cellulose-dextrin medium for identifying cellulose organisms in soil. — *Proc. Soil Sci. Soci. of America* 7:243. 1942.
6. Grassmann, W., Stadler, R. & Bender, R.: Zur Spezifität cellulose- und hemicellulose-spaltender Enzyme. — *Ann. d. Chemie* 502:20. 1933.
7. Jermyn, M. A. & Tomkins, R. G.: The chromatographic examination of the products of the action of pectinase on pectin. — *Biochem. J.* 47:437. 1950.
8. *Methodes of analysis of the association of agricultural chemists.* — 6. ed. p. 850. Washington 1945.
9. Levinson, H. S., Mandels, G. R. & Reese, E. T.: Products of enzymatic hydrolysis of cellulose and its derivatives. — *Arch. Biochem. Biophys.* 31:351. 1951.
10. Norman, A. G.: *The biochemistry of cellulose, the polyuronides, lignin etc.* — Oxford 1937.
11. O'Dwyer, M.: The structure of the hemicelluloses of the wood of English oak. iv. The structure of hemicellulose A. — *Biochem. J.* 33:713. 1939.
12. Partridge, S. M.: Filter-paper partition chromatography of sugars. 1. General description and application to the qualitative analysis of sugars in apple juice, egg white and foetal blood of sheep. — *Biochem. J.* 42:238. 1948.
13. Partridge, S. M.: Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. — *Nature* 164:443. 1949.
14. Phaff, H. J.: The production of exocellular pectic enzymes by *Penicillium chrysogenum*. I. On the formation and adaptive nature of polygalacturonase and pectinesterase. — *Arch. Biochem.* 13:67. 1947.
15. Pigman, W. & Goepp, R. M.: *Chemistry of the carbohydrates.* — New York 1948.
16. Pigman, W.: *Cellulases, hemicellulases, and related enzymes.* — Sumner & Myrbäck: *The enzymes* I:725. New York 1951.

17. Pringsheim, H.: Über den fermentativen Abbau der Cellulose. — *Z. f. physiol. Chem.* 78: 266. 1912.
18. — & Kusenack, W.: Über Lichenin und die Lichenase. — *Z. f. physiol. Chem.* 137: 265. 1924.
19. Stiles, H. R., Peterson, W. H. & Fred, E. B.: A rapid method for the determination of sugar in bacterial cultures. — *J. Bact.* 12: 427. 1926.
20. Voss, W. & Butter, G.: Über die fermentative Spaltung von Xylan. — *Ann. d. Chem.* 534: 161. 1938.
21. Waksman, S. A. & Stevens, K. R.: A system of proximate chemical analysis of plant materials. *Ind. Eng. Chem. Anal. Ed.* 2: 167. 1930.
22. Weidenhagen, R.: Die Zytasen. — Baman & Myrbäck: *Die Methoden d. Fermentforschung* p. 1910. 1941.
23. Weihe, H. D. & Phillips, M.: Hemicelluloses of wheat straw. — *J. Agr. Res.* 60: 781. 1940.

Methods for Determining Stages of Development in Barley and Oats

By

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Introduction

In an early stage of development barley and oats are rather susceptible to treatment with the growth regulators used in weed control. The damage seems to depend mainly on the developmental stage of the growing point or the ear, but as yet there is no method by which the susceptible stage can be determined. The aim of the present investigation was to work out such a method.

In an earlier investigation the number of leaves were counted and the length of the ear measured under a microscope (Andersen and Hermansen 1950). However, length measurements require much time, and probably will be less accurate than a determination of the morphological stages in the development of the ear. On the basis of this development a scale of growth stages was set up. As ear analysis cannot be made in the field, whereas leaves are readily counted, an attempt was made to find a relation between number of leaves and stage of ear development.

Two phases exist in the development of the shoot apex, vegetative and reproductive (Bonnett 1935, Evans and Grower 1940, Sharman 1946). In the vegetative phase only leaf primordia are being formed on the 'growing point' or 'shoot apex'. The reproductive phase sets in when spikelet primordia are being formed. The growing point is now an inflorescence, an 'ear'. In order to simplify reading the terms 'growing point' and 'shoot apex' will not be

used very often in the present paper. The words 'ear', 'spike', and 'panicle' will be used as terms for both the growing point and the inflorescence. The word 'ear' will be used most frequently.

Literature

Gregory and Purvis (1938) used a scale of conventional units in their work with vernalization of rye. The stage of maturity actually reached by the ear was expressed by units, which were derived from the duration in days of the successive stages of morphological development of ears from normal plants. However, not much use was made of this scale. Feekes (1941) introduced a scale of growth stages in wheat and described 11 stages with sub-stages from emerging to ripening. During the periods of tillering and stem extension this scale is based on the morphological development of leaves, tillers, and nodes. From the time of heading the scale is based on the development of the spike. Feekes used the scale in vernalization experiments, and this scale is now used in work with disease assessment in England. In an earlier paper (Andersen and Hermansen 1950) we advanced the term 'visible leaves'. We found in one variety of barley and one variety of oats a rather good correlation between number of leaves and length of ear, but a few analyses from other varieties indicated that varietal differences exist.

As a basis for the method presented here Bonnett's papers have been of the greatest value. He described the morphological development of the ear of barley, of wheat, and of oats in three papers (Bonnett 1935, 1936, and 1937). Evans and Grower (1940) devoted their studies to the morphology of the ear in the grasses, and Lermer and Holzner (1888) and Sharman (1945, 1946) have mainly considered the anatomical development of the growing point and the young inflorescence.

Plan of experiments

The present investigations were carried out in connection with spraying experiments, in which 2,4-D was applied at different times. Most of the experiments were located at Albertslund, the experimental farm of the Agricultural College. In 1949 only one variety of barley (Rigel) and one variety of oats (Stål) were sown. In 1950 and 1951 4 varieties of barley were sown: Freja, Maja, Alfa, and Rigel, and 4 varieties of oats: Minor, Rex, Opus, and Stål. In 1951 an experiment with barley was placed at Aarhus. In that experiment the same 4 varieties were used.

In 1949 and 1950 the cereals were sown about April 1, which is the normal time in Denmark. In 1951 sowing was very late, April 25 at Albertslund and April 23 at Aarhus.

In 1949 plants were analysed at 12 different dates for barley and 15 for oats, the first date was very soon after emergence, the last just before heading. In 1950 and 1951 plants were analysed at 7 different dates. The first analysis of barley was made when the plants averaged 1.5 leaves, and the last when the average was between 4.0 and 4.5. The analysis of oats took place somewhat later. The first analysis was made when the plants averaged 2.5 leaves, and the last when the average was about 5.5 leaves.

Number of leaves

The leaves were counted on 50 plants and the average taken. Only the main stems were used for the analysis, as the plants generally average 1.5—2.0 spikes. The main stems are most important, and it is not possible to see whether the tillers will be fertile or sterile. Plants obviously much damaged by insect attacks and therefore considerably below average, were discarded.

Only »visible» leaves were counted. That term was introduced in an earlier paper (Andersen and Hermansen 1950). It means that no new leaf is counted before its length in centimeters is equivalent to one half of its number in the scale. E.g. the third leaf was not counted before it had a length of 1.5 cm from the ligule of the first or the second leaf. Leaves above that length may in most cases be counted very easily, but it is difficult to count smaller leaves without a careful examination of the plants.

The average number of leaves of 50 plants did not increase at a constant rate. Relatively much time was required for a small increase when that number was close to a whole figure. Half-way between two whole figures it increased rapidly, and many plants developed a new leaf on the same day. In a few cases, when nearly all 50 plants had the same number of leaves, counting the number of leaves showed no increase at all for two or three days. The sudden increase in leaf number is of course most pronounced in an evenly developed crop, where most plants will produce a new leaf at the same time. The number of leaves shows the stage of development most accurately when about 25 plants have one number of leaves and about 25 plants another number.

In most of the analyses the 50 plants would fall into two groups differing only one unit in leaf count. The number of plants in a third group very seldom exceeded 3. In calculating the standard error by means of the formula of the binomial distribution in 50 plants the following figures were obtained:

Number of leaves (decimals)10 or .90	.30 or .70	.50
Standard error	± 0.043	± 0.065	± 0.071

Stage of development of ear

When the number of leaves had been counted, the 15 smallest and the 15 largest plants were discarded. Of the others, which were bottled in alcohol, 12 plants of barley and 15 of oats were used for ear analysis. The ears were dissected from the plants under the microscope, and the stage of development determined by means of two scales, worked out for that purpose (Tables 1 and 2).

Table 1. *Barley. Stage of the growing point and the spike.*

Stage	Developmental morphology
0	The growing point hemispherical and still placed in the kernel.
1	With or without a single leaf primordium.
2	2 to 3 leaf primordia (single ridges).
3	4 to more leaf primordia.
4	The leaf primordia are being transformed to primordia of the central spikelets (double ridges). The apices of the spikelet primordia are <i>stumpy square-cut</i> .
5	The apices of the central spikelets are <i>hemispherical</i> .
6	Formation of side spikelets.
7	Lemma visible on 1 to 6 spikelets on each side of the spike.
8	Lemma visible on 7 to more spikelets. Anthers visible in the most developed spikelets.
9	The tip of the awn reaches the spikelet above on 1 to 6 spikelets in each side.
10	The tip of the awn reaches the spikelet above on 7 to more spikelets.
11	The awns longer than the spike.
12	Palea longer than stamens in spikelets in the middle of the spike.
13	Anthesis. (Or heading, awns just showing).

Table 2. *Oats. Stage of the growing point and the panicle.*

Stage	Developmental morphology
0	The growing point hemispherical.
1	With or without a single leaf primordium.
2	2 to more branch initials of first order.
3	Branches of second order formed.
4	The lemma on first flower in the top spikelet forms a collar.
5	Anthers in first flower.
6	The empty glumes enclose half the top spikelet and cover it where it is widest.
7	The top spikelet covered by empty glumes.
8	Most spikelets covered by empty glumes.
9	All spikelets covered by empty glumes.
10	Heading. Panicle escaping through split of sheath.

The stage reached is characterized by morphological signs. These signs must be so chosen that they are easily recognized and the change from one stage to another very distinct. In most cases no mistake can be made, but the change from stage 1 to stage 2 may present difficulties. It is often doubtful whether a leaf primordium in the top is visible or whether a leaf primordium at the base is already a leaf. For this distinction a leaf primordium means a crescent-shaped ridge with a horizontal edge. When the leaf-primordium



Fig. 1. *Photomicrographs of some stages in ear development.*

- 1) Barley, stage 3. l, two large collar-shaped leaves at the base; lp, leaf primordia.
- 2) Barley, early stage 4. l, leaf; lp, leaf primordia; sp, spikelet primordia.
- 3) Barley, late stage 5 with many spikelets.
- 4) Oats, stage 3. b₁, branch of first order; b₂, branch of second order; g, empty glume.
- 5) Oats, stage 4. g, glume; le, lemma; f₁, first flower; f₂, second flower.
- 6) Oats, stage 5. g, glume; le, lemma; a, anthers; f₂ and f₃, second and third flower.

becomes collar-shaped with an edge neither horizontal nor vertical, it is no longer considered a leaf primordium but a leaf.

A few stages are shown in Fig. 1. The photos are not very distinct as the plants had been stored for some time in alcohol. The reader is advised at this point to make himself familiar with Bonnett's excellent photomicrographs (Bonnett 1933 and 1937).

The time required for the successive stages of development was found to

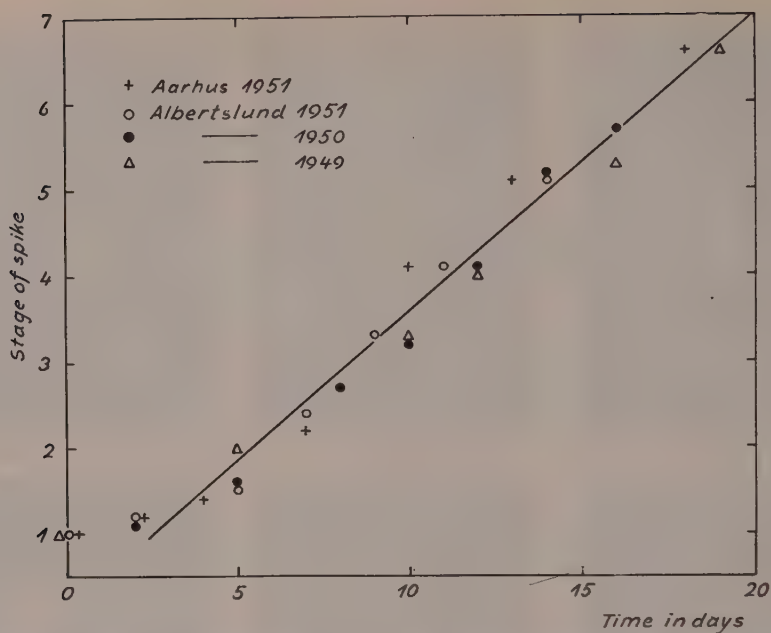


Fig. 2. Barley. Stage of development of the spike at successive dates in 4 experiments. Figures from one variety (Rigel) grown in 1949, and average figures from 4 varieties grown in 1950 and 1951.

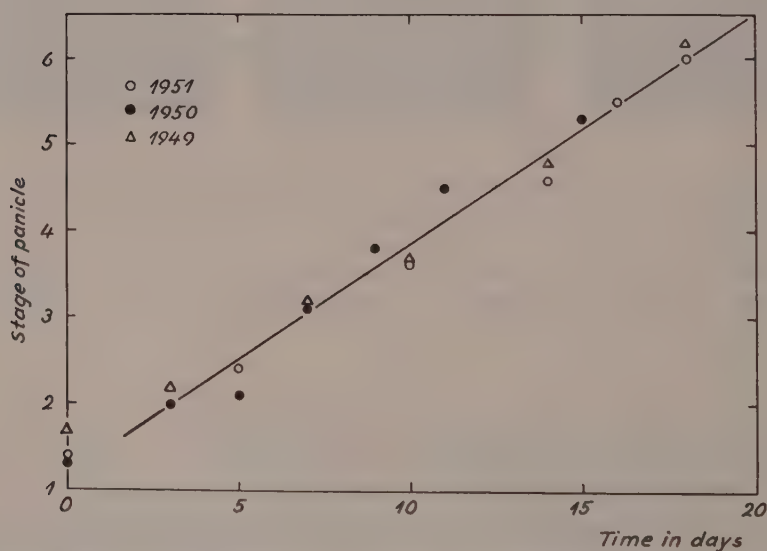


Fig. 3. Oats. Stage of development of the panicle at successive dates in 3 experiments. Figures from one variety (Stål) grown in 1949, and average figures from 4 varieties grown in 1950 and 1951.

be about 3 days for barley and 4 for oats (Figs. 2 and 3). Nearly the same time was necessary for development of each one of the stages between 2 and 7. Therefore, within these limits it is unnecessary to state the number of ears in each stage, the average figures can be used.

Stage 1 in barley is of a long duration, but also here an average figure may be used since ears of higher stages than 2 were very rare in such cases. Only in analyses from 1949 were found ears of a higher stage than 8 in oats and 7 in barley, but it seems likely that the duration of such higher stages will be 2—3 days longer than of those below 7. When stage 7 had been passed, the ears in an analysis always showed two stages only.

The ears from analyses between stage 2 and stage 7 belonged in most cases to two stages only, even if a single ear might belong to a third. Analyses in which several ears belonged to a third stage were rare. However, the variation found for ears does not show all the variation possible, because plants of average size were selected for analysis. This selection reduces variation.

Correlation between number of leaves and stages of ear development

This correlation is rather complicated, since the ear did not start its growth at the same number of leaves in all varieties and in all the years in question. Correlation in the different years is shown in Figs. 4 and 5. Average figures of all 4 varieties were used so that the diagrams should not be too complicated.

The barley spike had reached a high stage of development very early at Albertslund in 1949 and in the experiment located at Aarhus in 1951 (Fig. 4). A corresponding stage of development was not reached at Albertslund in 1950 and 1951 until the plants had developed one leaf more. For the same leaf number the oat panicle was in a lower stage of development in 1949 than in 1950 and 1951 (Fig. 5). In 1949 however, only the variety Stål was grown. The difference between the crops grown at Albertslund in 1950 and 1951 is smaller, but both the barley spike and the oat panicle were for the same leaf number in a higher stage of development in 1951 than in 1950.

Differences in correlation were mainly due to an earlier or later start of growth of the ear. When growth was started, it continued at approximately the same rate. An exception from the rule was oats in 1949 and to some extent barley at Aarhus 1951. Different correlations in varieties also are mainly due to an earlier or later start of ear growth, although differences in the stage of ear development are a little greater with a high number of leaves than with a low number.

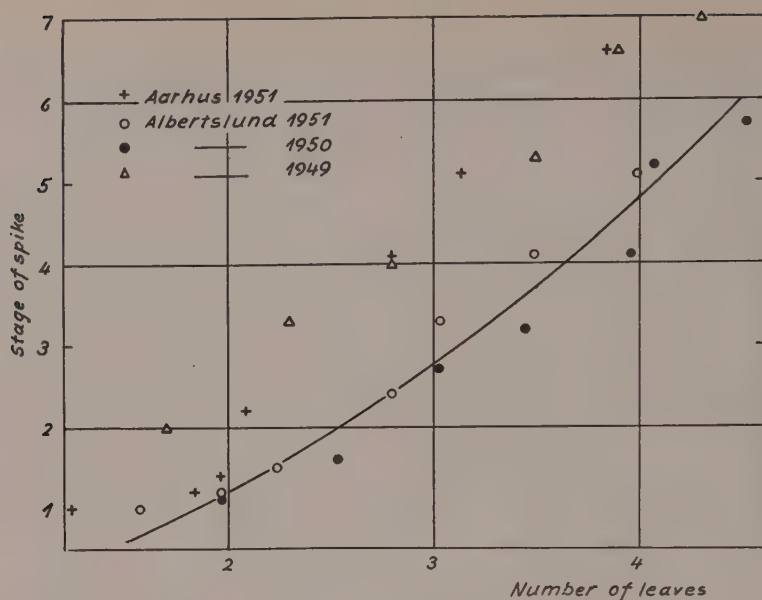


Fig. 4. Barley. Correlation between stage of spike development and number of leaves in 4 experiments. Figures from one variety (Rigel) grown in 1949, and average figures from 4 varieties grown in 1950 and 1951. The curve is based on the figures from the varieties Maja and Rigel grown at Albertslund 1950 and 1951.

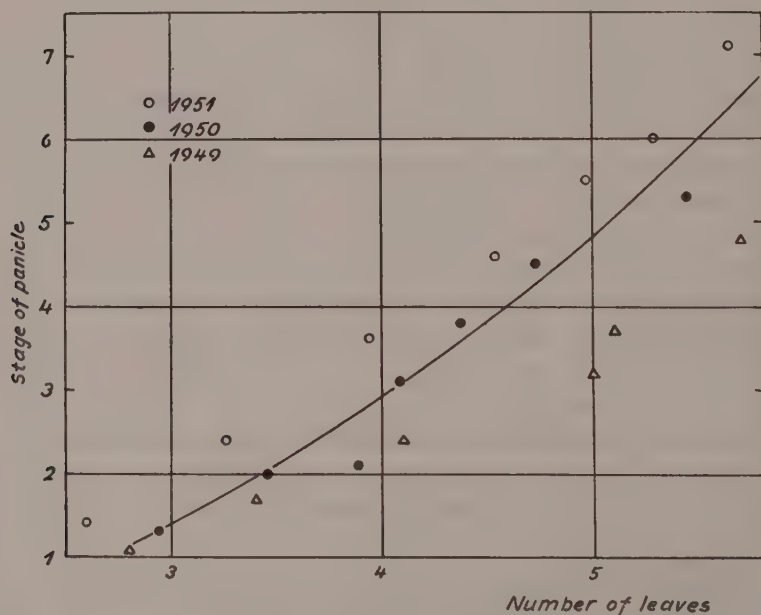


Fig. 5. Oats. Correlation between stage of panicle development and number of leaves in 3 experiments. Figures from one variety (Stål) grown in 1949, and average figures from 4 varieties grown in 1950 and 1951. The curve is based on figures from the varieties Opus and Stål grown in 1950 and 1951.

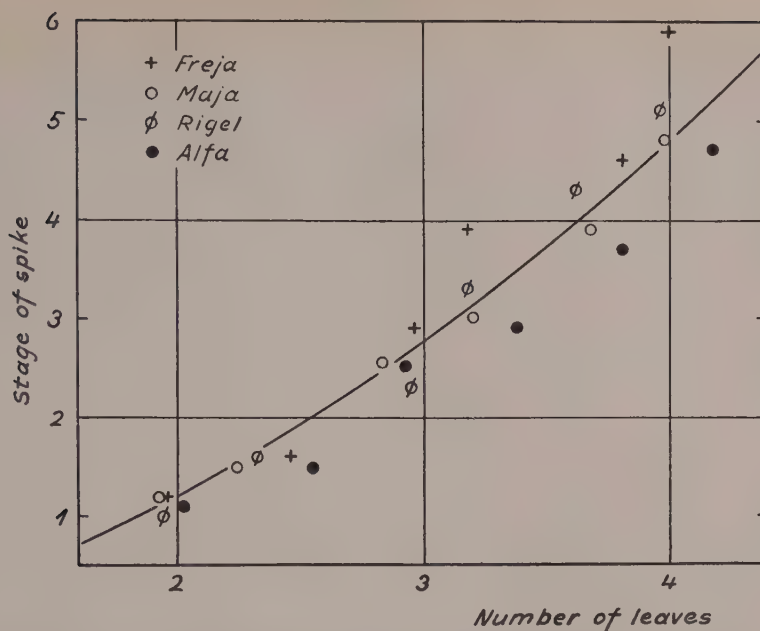


Fig. 6. Barley. Correlation between stage of spike development and number of leaves in 4 varieties grown at Albertslund in 1950 and 1951. Each figure is an average of one analysis from 1950 and one from 1951. The curve is based on the figures from Maja and Rigel.

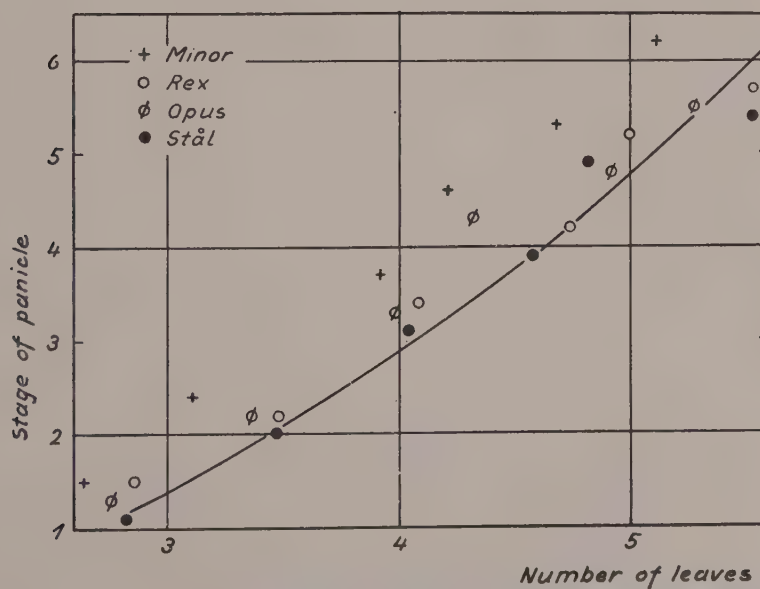


Fig. 7. Oats. Correlation between stage of panicle development and number of leaves in 4 varieties. Each figure is an average of one analysis from 1950 and one from 1951. The curve is based on the figures from Opus and Stål.

The correlation in different varieties is shown in Figs. 6 and 7. In order to eliminate variations from year to year the following procedure was used: In two successive years, 1950 and 1951, on 7 corresponding dates, each time when the plants were in the same stage of development, analyses were made. Six of the corresponding pairs agreed well and were used for calculating average figures. The seventh pair was discarded. The figures show that Freja is an early variety and Alfa a late one, but no difference is apparent between Maja and Rigel. The deviation of Minor is obvious, but no significant difference is found between Rex and Opus and Stål, although Stål seems to be a little later than Rex.

Discussion

The stage of development of cereals may be determined in several ways. Some methods may be used in the field, others in the laboratory only.

Measurement of the length of the ear and determination of the stage of ear development must be carried out at the laboratory using a microscope. In an earlier work only the length of the ear was measured (Andersen and Hermansen 1950). In the present investigation both length and stage of development were determined on 700 barley spikes and 700 oat panicles, and a good correlation is found between length and stage of ear. Both methods are useful in experiments with growth regulators, but for practical reasons a determination of the stage of ear development is preferable for future work. By means of this method it is possible to analyse more than twice as many ears as by length measurement.

In table 3 the length of the ear in different stages is shown. The barley spikes in stage 3 and 4 were of about equal length, as the change from stage 3 to stage 4 took place very early in 1951 and very late in 1950. On the whole the table makes it possible to compare length and stages of ear development.

The stage of development can be determined in the field by means of the height of the plant. This is an easy but unsatisfactory method. The final height depends on year and variety, and the method is only useful in a very young crop. A better, and still easy method is leaf count; sampling of 50 plants and counting the leaves can be carried out in less than half an hour.

In an earlier investigation (Andersen and Hermansen 1950) a good correlation was found between number of leaves and length of the ear, at least within the same variety. Unfortunately, the present investigation has shown that the ear did not always start growth when the plants had the same number of leaves. This places growth curves differently in the diagrams. Thus in the barley experiments at Albertslund in 1949 and at Aarhus in

Table 3. *Average length in mm of barley spikes and oats panicles. Measurements from a total number of 700 barley spikes and 700 oat panicles 1949—1951.*

Stage of development of the ear	1	2	3	4	5	6
Barley	0.27	0.40	0.56	0.62	0.98	1.24
Oats	0.23	0.41	0.69	0.98	1.37	1.97

1951 the ear was in a stage, which in the other experiments did not appear until the plants had developed nearly one leaf more (Fig. 4). No explanation of this fact can be given at present. It is known that long days favour the development of the inflorescence in grasses (Gregory and Purvis 1938, Evans and Grower 1940, Whyte 1945, Cooper 1950), but other factors seem to be of importance too. The barley crops were sown at approximately the same date in 1949 and 1950, so that the day-length has been very nearly the same in the two years. However, if the claims to accuracy are not too high, the number of leaves can be used when the plants have less than 6 leaves. Leaf count on older plants require much time, as the first leaves die and may be difficult to recognize.

The author has not found Feekes' method (Feekes 1941) easy to apply to young plants, but from Feekes' stage 8 and to ripening the method is very easy and can be applied to all cereals. The author would therefore suggest two methods for determination of stage of development in spring sown cereals, one for field and one for laboratory use. In the field the number of leaves indicate the stage of development as long as the number is below 6; after that Feekes' method may be used. In the laboratory the stage of development of the ear in barley and oats can be determined until anthesis or heading; after that Feekes' method may be used in the laboratory too.

In many kinds of field experiments with cereals the same treatment is applied at different times, and in most cases only the date of treatment is stated. A determination of the stage of development would be more rational.

Summary

A method is described by which ears (or shoot apices) of barley and oats can be classified into stages of morphological development. By that method the stage of development can be determined rather easy.

A correlation between stage of development of ear and number of leaves was sought. Such a correlation has been found in part. Variety as well as

year will influence the final results, but a determination based on the counting of the leaf number is justified if no high degree of accuracy is required.

It is suggested that the stage of development in young plants should be determined by leaf count or by examination of stage of ear development, and in older plants by Feekes' method.

References

- Andersen, S. & Hermansen, J. E.: Effect of hormone derivatives on cultivated plants II. Spraying of cereals with 2,4-D and 4K-2M at different dates. — Kgl. Vet. & Landbohøjskoles Årsskr. København 1950: 141.
- Bonnett, O. T.: The development of the barley spike. — Jour. Agric. Res. 51: 451. 1935.
- The development of the wheat spike. — Jour. Agric. Res. 53: 445. 1936.
- The development of the oat panicle. — Jour. Agric. Res. 54: 927. 1937.
- Cooper, J. P.: Day-length and head formation in the ryegrasses. — Jour. Brit. Grassl. Soc. 5: 105. 1950.
- Evans, M. W. & Grower, F. O.: Developmental morphology of the growing point of the shoot and the inflorescence in the grasses. — Jour. Agric. Res. 61: 481. 1940.
- Feekes, I. W.: De tarwe en haar milieu. — Verslagen van de technische tarwe commissie 17. 1941. (Engl. summary).
- Gregory, F. G. & Purvis, O. N.: The vernalization of excised mature embryos, and of developing ears. — Ann. Bot. n.s. 2: 237. 1938.
- Lermer & Holzner: Beiträge zur Kenntnis der Gerste. — München 1888.
- Sharman, B. C.: Leaf and bud initiation in the gramineae. — Bot. Gaz. 106: 269. 1945.
- The biology and the developmental morphology of the shoot apex in the gramineae. — New Phytologist 46: 20. 1946.
- Whyte, R. O.: Crop production and environment. — London 1946.

The Persistence of Aquatic Plants to Extreme pH Values

By

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The influence of the pH of the soil on terrestrial plants asserts itself both directly and indirectly. Generally the indirect effects of this factor are more evident. Here the influence of pH on the uptake of necessary elements, e.g. Fe, and of poisonous elements, e.g. Al, is to be mentioned.

Similarly, the indirect influence of pH in the surrounding water on submersed plants is in nature usually more evident than the direct influence.

A great many submersed flowering plants, as *Helodea canadensis*, *Ceratophyllum demersum*, and *Myriophyllum spicatum*, are in nature found in alkaline water only (Iversen 1928). By culture experiments with *Helodea canadensis* and *Ceratophyllum demersum* it was, however, shown (Steemann Nielsen 1944) that pH within the amplitude 4.5 to 8.2 has no appreciable influence on growth when only one of the carbon sources for the photosynthesis (free CO_2 or HCO_3^- -ions) was in excess. The fact that the two species in nature are found in alkaline water only, is due to the contents of assimilable carbon being much lower in acid than in alkaline water. The reason for this is that the amount present of one of the carbon sources — HCO_3^- -ions — decreases with decreasing pH.

Aquatic flowering plants, therefore, must be able generally to maintain a concentration of hydrogen ion in the protoplasm independent of pH in the surrounding water. The uptake of the two different carbon sources — free CO_2 and HCO_3^- — is further, in these plants, independent of pH in the surrounding water (pH amplitude about 4 to 10). The rate of photosynthesis in *Myriophyllum spicatum* is thus independent of pH when the carbon source (free CO_2 or HCO_3^-) is in excess as well as when the concentration

of one of these carbon sources is the limiting factor for photosynthesis (Steemann Nielsen 1947, pag. 50—54).

The surrounding water must, however, be a balanced solution of different salts, as the rate of photosynthesis at low concentrations of HCO_3^- -ions otherwise depends on pH. On the other hand the uptake of free CO_2 is, in the species mentioned, independent of pH both in solutions of one single salt and in balanced salt solutions.

The ability to tolerate water of different pH seems to be much less in the majority of green algae than in the higher flowering plants. The green alga *Scenedesmus quadricauda* is an exception (cf. Österlind 1949). Steemann Nielsen 1944 thus found a pronounced pH dependence in a *Stigoclonium* sp. although free CO_2 was in excess at all pH values. No growth was found at pH 3.5 and 4.5. At pH 5.5 growth could just be observed. At pH 6.5 the growth was still rather insignificant. Only at pH 7.5 and 8.0 a vigorous growth was found.

In another green alga, *Cladophora insignis*, a rather similar pH dependence was found (Steemann Nielsen 1947, pag. 54—56). At pH 6.4 the rate of photosynthesis with free CO_2 in excess was reduced about 20 per cent. in comparison with experiments at pH 7.4. At pH 5.6 the rate was nearly constant during the first hour, although reduced in comparison with the rate at pH 7.4. After about 2 hours the photosynthesis nearly ceased.

The presence of neutral salts (a balanced salt solution) to some extent helps the plant to endure acid water. The above-mentioned experiments at pH 5.6 were made in a balanced solution of neutral salts. In other experiments made at the same pH but without addition of neutral salts photosynthesis stopped already after about half an hour and the plants fell to pieces.

Emerson and Green 1938 have investigated the green algae *Chlorella vulgaris* and *Chlorella pyrenoidosa*. In the pH-range 4.6 to 8.9 they found no influence on the rate of photosynthesis when free CO_2 was in excess. They made no experiments concerning the pH influence when CO_2 was the limiting factor. Such experiments on *Chlorella pyrenoidosa* have been made by Österlind (1950) who in 0.1 % of CO_2 found good growth at high pH values, decreasing linearly with decreasing pH. In 0.1 % of free CO_2 the carbon source is limiting at all pH values as HCO_3^- -ions can not be used in the photosynthesis of this plant (shown by Österlind, too). Also at higher concentrations, where this factor does not limit photosynthesis, Österlind found decrease in growth with decreasing pH. As will be shown below, these results do not necessarily contradict those of Emerson and Green. Photosynthesis and growth are not identical processes although in autotrophic plants the latter depends on the former.

One of the reasons why the dependence on pH in photosynthesis and

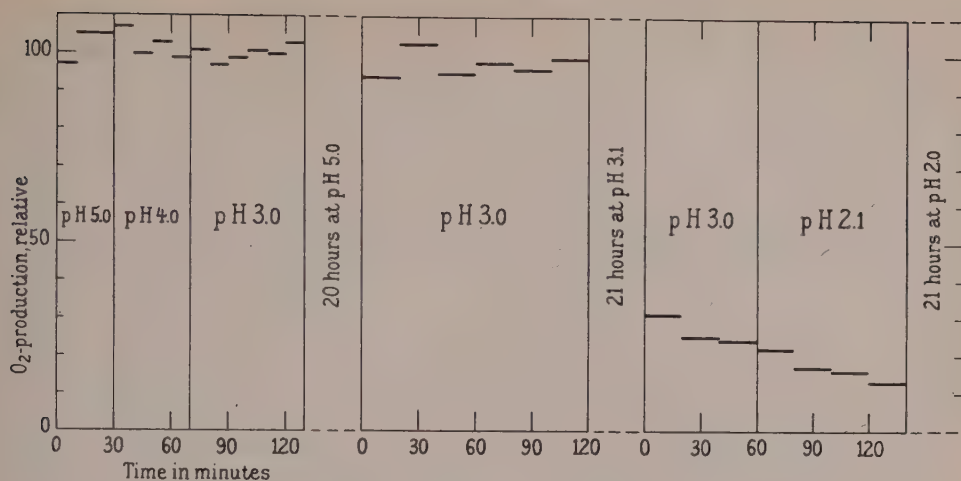


Fig. 1. *Fontinalis dalecarlica* at 15,000 lux (see text).

growth is not necessarily the same, is the time factor. Experiments on photosynthesis are mostly of short duration only. On the other hand growth experiments must take some time. The significance of the time factor for the toleration of extreme pH values will be illustrated through experiments with the two water mosses *Fontinalis dalecarlica* and *Fontinalis antipyretica*.

Fig. 1 illustrates some experiments on the rate of photosynthesis in *Fontinalis dalecarlica* when pH varies between 5.0 and 2.0. According to Sørensen 1948 the pH amplitude in nature is 4.2–6.6. The method for measuring the rate of photosynthesis was the same as that described by Steemann Nielsen 1942. Photosynthesis was measured by means of the oxygen production. The oxygen was determined by means of a Winkler analysis. The water was vigorously stirred during the experiments. pH was measured electrometrically with the glass electrode. The water for the experiments had a concentration of free CO₂ of 1 millimole per litre which is sufficient for producing maximum rate of photosynthesis in the *Fontinalis* species (Steemann Nielsen 1952). It was made by mixing glass-distilled water saturated with CO₂ with a solution of 0.08 gr. CaCl₂, 0.02 gr. K₂SO₄, and 0.01 gr. NaCl in 1 litre glass-distilled CO₂-free water. A definite pH was produced by adding HCl.

Fig. 1 clearly shows that the rate of photosynthesis at optimum concentration of free CO₂ is unaltered in the pH-range 5–3 if the experimental time at the lowest pH is not too long. No influence on the rate of photosynthesis after one hour at pH 3.0 was observed. No indirect influence was observed either. The rate of photosynthesis was still unaltered the next day. The plants had been kept at pH 5.0 during the night. Nor do two hours at pH 3.0 in-

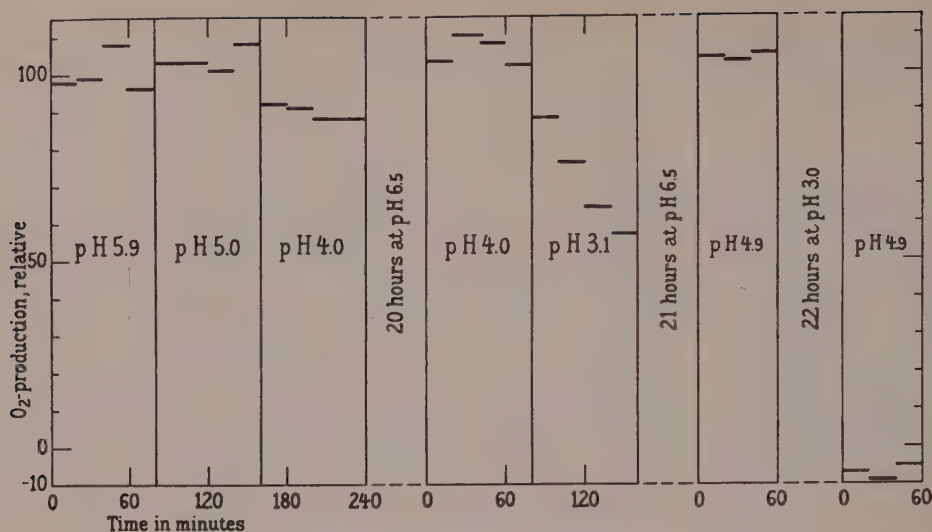


Fig. 2. *Fontinalis antipyretica* at 15,000 lux (see text).

fluence *Fontinalis dalecarlica*. After 23 hours at pH 3.0–3.1 the rate of photosynthesis is, however, strongly reduced, but the plants had still a fresh green appearance. When the plants were transferred to water having a pH of 2.1 the rate of photosynthesis still decreased. After 80 minutes the appearance of the plants was still unaltered. The plants had, however, almost completely lost the green colour after 21 hours at pH 2.0.

Similar experiments were made with *Fontinalis antipyretica* (fig. 2). The ability to endure low values of pH was much less in this species. This is in accordance with the behaviour in nature. The pH amplitude for this species in nature according to Sørensen 1948 is 5.5–8.2. The rate of photosynthesis decreases from the first moment when the plants are transferred to pH 3.1. After 80 minutes at this pH the rate has decreased to about 50 per cent. After 22 hours at pH 3.0 the plants had nearly completely lost the green colour.

Although the influence of pH 3.1 for 80 minutes was very distinct, *Fontinalis antipyretica* recovered completely when transferred to less acid water. This is clearly demonstrated in the experiments illustrated in fig. 2.

The first experiments at pH 4.0 (fig. 2) seem to indicate an injurious effect already at this pH. The decrease during an hour at pH 4.0, however, was only slight if any, and after 20 hours at pH 6.5 no decrease in the rate of photosynthesis was observed during 80 minutes at pH 4.0.

The different behaviour of the two *Fontinalis* species at low pH gives an excellent explanation why in more acid localities only *Fontinalis dalecarlica*

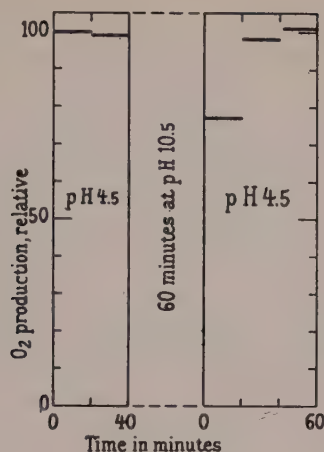


Fig. 3. *Fontinalis dalecarlica* at 15,000 lux (see text).

is found. In contradistinction to *Fontinalis antipyretica* this species is not found in alkaline water. Some experiments were therefore made to show if *Fontinalis dalecarlica* is specially sensitive to high pH values (high concentrations of OH⁻ ions). This does not, however, seem to be the case. Fig. 3 shows that the rate of photosynthesis at optimum concentration of free CO₂ after one hour's treatment at pH 10.5 is still unaltered. Treatment at pH 10.5 of much longer duration than one hour is likely to have a detrimental effect on the species. This, however, is without ecological significance as the OH⁻-concentration at pH 10.5 is about 1000 times as high as that found in water with sufficient contents of free CO₂, the only carbon source for photosynthesis in this plant (Steemann Nielsen 1951).

pH increases in some very eutrophic lakes during the summer to values between 10 and 11. This is caused by the enormous photosynthetic activity of the plankton algae using HCO₃⁻ ions whereby OH⁻ ions are released. It was thus found in the Danish lake Søllerød Sø during investigations in 1944 that pH was the real limiting factor for the production of phytoplankton at the height of summer. A publication on the chemistry, plankton production, and fishery biology in this lake is under preparation by K. Erik Jensen, C. V. Otterstrøm, and the present writer. The question of the influence of pH on plankton production will be thoroughly discussed in this publication. A single detail from these investigations is to be mentioned here.

Surface water collected in the lake in the evening of August the 17th had a rather considerable content of plankton algae. No photosynthetic activity could, however, be demonstrated in these algae when they were exposed to light. The algae had died. The saturation of oxygen in the surface water was 221 per cent., a saturation which is only possible at the surface if there has

been a considerable rate of photosynthesis during the day, thus indicating a very recent death of the algae. The oxygen in the surface water would otherwise have been interchanged with the atmosphere. pH was not measured at the surface on the evening of August the 17th, but the next morning — at 11 o'clock — pH was 10.1. This is not a particularly high value for pH, which the evening before without doubt was much higher. pH at the surface decreases during the night, partly because of absorption of CO_2 from the atmosphere, partly because of mixing of surface water with water from deeper layers due to the cooling of the surface water during the night. Without doubt the high pH brought about by the photosynthesis of the algae during the day causes the death of the algae.

Besides decreasing pH the vertical mixing of the water masses during the night also carries living plankton algae to the surface. Although in a bright and calm period at the height of summer all plankton algae just near the surface die during the daytime, life here is able to start again every morning.

Summary

The water moss *Fontinalis dalecarlica* will for hours persist pH values between 3.0 and 10.5. No reduction in the maximum rate of photosynthesis is observed after two hours at pH 3.0. A significant, although reduced rate of photosynthesis was still found after 23 hours at this pH. The appearance of the plants was still quite normal.

Fontinalis antipyretica is momentarily affected by pH 3.0. When transferring specimens in which the rate of photosynthesis is reduced through treatment at pH 3.0 to higher pH they will recover completely.

High pH values caused by the photosynthetic activity of plankton algae may be the real limiting factor for production in some very eutrophic lakes.

References

- Emerson, R. and Green, L.: Effect of Hydrogen-Ion Concentration on *Chlorella* Photosynthesis. — *Plant Physiology* 13:157. 1938.
- Iversen, J.: Studien über pH-Verhältnisse dänischer Gewässer und ihren Einfluss auf die Hydrophyten-Vegetation. — *Bot. Tidsskr.* 40:277. 1929.
- Sørensen, H.: Studies on the Ecology of Danish Water- and Bog Mosses. — *Dansk Bot. Ark.* 12, Nr. 10. 1948.
- Steemann Nielsen, E.: Der Mechanismus der Photosynthese. — *Ibidem* 11, Nr. 2. 1942.
- Dependence of Freshwater Plants on Quantity of Carbon Dioxide and Hydrogen Ion Concentration. Illustrated through Experimental Investigations. — *Ibidem* 11, Nr. 8. 1944.

- Photosynthesis of Aquatic Plants with special Reference to the Carbon Sources. — Ibidem 12, Nr. 8. 1947.
- Experimental Carbon Dioxide Curves in Photosynthesis. — Physiol. Plant. 5: 145. 1952.
- Österlind, S.: Growth Conditions of the Algae *Scenedesmus quadricauda* with special reference to the Inorganic Carbon Sources. — Symb. Bot. Upsal. 10, Nr. 3. 1949.
- Inorganic Carbon Sources of Green Algae. I. Growth Experiments with *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*. — Physiol. Plant. 3: 353. 1951.

Hans Fitting zum 75. Geburtstag gewidmet

Die Respiration von gesunden und viruskranken Zuckerrüben

Von

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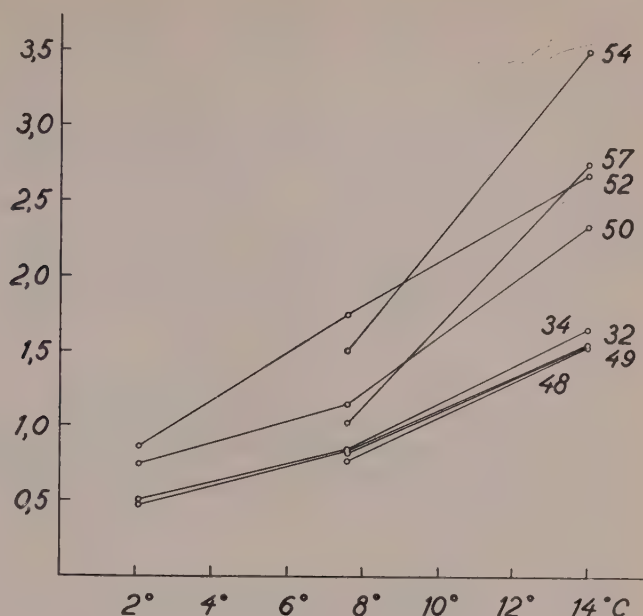
Die Verluste an Trockenstoff bei der Aufbewahrung von Zuckerrüben betragen grosse Werte. Die durch die Atmung verursachten Trockenstoffverluste sind wahrscheinlich von Sorte zu Sorte verschieden. Wir haben bisher nur von einer Sorte die Atmung untersucht und dabei gefunden, dass es einen grossen Unterschied zwischen gesunden und viruskranken Rüben gibt.

Für Versuche wurden Zuckerrüben *Beta vulgaris esculenta altissima*, Stamm Maribo N, verwendet. Die Rüben wogen 400—550 g. Zwischen den Versuchen wurden die Rüben so feucht gehalten, dass der Frischgewichtsverlust nur ungefähr 0,5 g pro Tag betrug, sodass der Trockenstoffprozent der Rüben sich konstant hielt. Die Atmung wurde auf das für den Versuchszeitpunkt jeweilig bestimmte Frischgewicht berechnet. Es wurden teils ge-

Tabelle 1. Verlust an Trockenstoff in g durch Atmung in 30 Tagen pro 100 kg Frischgewicht Zuckerrüben bei drei verschiedenen Temperaturen.

Gesunde Zuckerrüben			Virus-gelbsüchtige Zuckerrüben		
2,1°	7,6°	14,0°	2,1°	7,6°	14,0°
245 ± 12	357 ± 7	707 ± 20	301 ± 23	518 ± 32	1149 ± 65

Abb. 1. *Respiration von Zuckerrüben.* Ordinate: mg CO₂ pro 100 g Frischgewicht pro Stunde. Abscisse: Temperatur. Nr 32, 34, 48 u. 49 sind gesunde Rüben. Nr 50, 52, 54 u. 57 sind virus-gelbsüchtige Rüben.



sunde, teils mit Virusgelbsucht (Beta Virus 4) infizierte Rüben untersucht. Die Atmungsbestimmungen wurden mit der Luftstrommethode ausgeführt. Es war wichtig, dass die durchströmende Luft beinahe dampfgesättigt gehalten wurde; denn sonst transpirierten die Rüben so stark, dass ihre Temperatur niedriger war als die Temperatur des den Recipienten umgebenden Wasserbads. Jedes Rübenindividuum wurde für sich untersucht, gleich nach Aufnahme am 1. November und mit Zwischenräumen bis Anfang Februar. Die Atmung jeder einzelnen Zuckerrübe hielt sich während dieser 3 Monate ziemlich konstant. Der Unterschied in der Atmung zwischen den gesunden Zuckerrüben war gering; aber sie war bedeutend niedriger als die Atmung der virus-gelbsüchtigen (Abb. 1.) Alle gewonnenen Zahlen von 19 untersuchten Zuckerrüben, auch die von Rüben, deren Atmungswerte nicht in Abb. 1 berücksichtigt wurden, sind in Tabelle 1 zusammengestellt. In dieser Tabelle sind die Atmungswerte in Trockenstoffverlust pro 100 kg Frischgewicht pro 30 Tage umgerechnet und zwar wie folgt: Der Trockenstoff der Rüben hatte einen C-Gehalt von durchschnittlich 44,9 %. 100 g CO₂ entsprechen also einem Trockenstoffverlust von 60,75 g. Man sieht aus Tabelle 1, dass der Trockenstoffverlust durch die Atmung der gesunden und viruskranken Zuckerrüben bei 2,1° 245 g resp. 301 g pro 100 kg Frischgewicht pro Monat beträgt. Der mittlere Fehler, berechnet nach der Formel $\sqrt{\frac{\sum d^2}{n(n-1)}}$, zeigt,

dass der Unterschied nicht signifikant ist. Bei den höheren Temperaturen ist der Unterschied aber signifikant und sehr gross. Bei 14° ist die Atmung und der Trockenstoffverlust der viruskranken Zuckerrüben beinahe 1,8 mal so gross, als bei den gesunden Rüben. Der Trockenstoffprozent der Zuckerrüben war 20,5 %. Daraus lässt sich der prozentische Trockenstoffverlust durch die Atmung der Rüben pro 30 Tage berechnen. (Die Zahlen in Klammern gelten für die viruskranken Rüben): Bei $2,1^{\circ}=1,19\%$ (1,47 %), bei $7,6^{\circ}=1,74\%$ (2,52 %) und bei $14,0^{\circ}=3,44\%$ (5,60 %).

Zusammenfassung

Die Atmung von virus-gelbsüchtigen Zuckerrüben ist bei $7,6^{\circ}$ und 14° beträchtlich höher als die von gesunden Rüben derselben Sorte. Bei $2,1^{\circ}$ ist der Unterschied gering.

Photosynthese von *Anemone nemorosa*

Von

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(Eingegangen am 22. Februar 1952)

Die Frühlingspflanzen im Walde, die ihre Hauptassimilationszeit vor der Laubentfaltung der Bäume haben, sind weder ausgesprochene Lichtpflanzen noch ausgesprochene Schattenpflanzen. Die maximale Photosynthese und das Blattareal pro Bodenflächeneinheit sind kleiner als die entsprechenden Werte für ausgesprochene Lichtpflanzen, doch stehen sie diesen genähert, jedenfalls ist das der Fall für *Anemone nemorosa*, wie es aus dem folgenden zu ersehen ist.

In der Literatur ist das Problem der Photosynthese der Frühlingsflora in den sommergrünen Buchenwäldern der temperierten Zone von Lundegårdh (1925, 1949) erwähnt. Dieser findet für *Anemone nemorosa* bei 0,228 mm CO₂-Druck eine maximale apparente Photosynthese bei 20° von 5,2 mg CO₂, bei 15° 6,4 mg CO₂ und bei 10° 5,5 mg CO₂, alles pro 50 cm² Blattfläche (einseitig) pro Stunde. Bei einer Beleuchtungsstärke von 11000 Lux waren die Werte bei 20° 1,7 mg CO₂, bei 15° 3,0 mg CO₂ und bei 10° 3,1 mg CO₂, alles pro 50 cm² Blattfläche (einseitig) pro Stunde. Die Werte von Lundegårdh dürften etwas zu niedrig sein, besonders die Werte bei 11000 Lux. Auch Daxer (1934) arbeitete mit der Waldenbodenflora und hatte als Hauptversuchsobjekt *Anemone nemorosa*. Seine Tageskurven der Photosynthese zeigen, dass *A. nemorosa* stärkeres Licht besser auszunutzen vermag als Schattenpflanzen wie z.B. *Oxalis acetosella*, bei denen eine Zunahme der Lichtintensität ohne Einfluss auf ihre Photosyntheseintensität bleibt. Er findet am 29. April eine maximale Photosynthese einer nichtblühenden *Anemone* von 6 mg CO₂ pro 50 cm² Blattfläche einseitig gemessen pro Stunde.

Pflanzenmaterial und Standort. Das für die Versuche verwendete Pflanzenmaterial wurde jeden Morgen um 9 Uhr aus Charlottenlund (einem

Wald 8 km nördlich von Kopenhagen) geholt. Die für die Photosynthesebestimmungen erwählten Laubblätter wurden behutsam, um die Leitbündel nicht zusammenzudrücken, mit einer scharfen Rasierklinge abgeschnitten und der Blattstiel wurde sofort in eine kleine mit Wasser gefüllte Vase gesteckt und diese wiederum in einer grossen mit feuchtem Filtrierpapier ausgelegten Petrischale angebracht. Auf diese Art und Weise blieben die Spaltöffnungen während des Transportes, der ca $\frac{1}{2}$ Stunde dauerte, und auch weiterhin geöffnet. Gleichzeitig mit dieser Probenentnahme wurden ca 20 g Anemoneblätter, bestimmt für Respirationsversuche, abgeschnitten und ebenfalls sofort in einem Glas mit Wasser angebracht. Die Beleuchtungsstärke gerade über der Anemonebewachung war vor Beginn der Laubentfaltung der Bäume 51 % des vollen Tageslichtes. Nur einmal wurde eine Probe von *A. nemorosa* aus einem Bestand, an der Lichtgrenze von dem Vorkommen von *Anemone*, entnommen. Dieser Standort hatte eine Beleuchtungsstärke von 12 %.

Die Beleuchtungsstärke um 12 Uhr Mittag ist im Freien im April durchschnittlich 32000 BJ-Lux, ungefähr 70 % der Beleuchtungsstärke unserer hellsten Monate Mai und Juni (Nach Messungen von P. Boysen Jensen, publiziert von Romose 1940).

Methodisches. Zur Bestimmung der Photosynthese wurde die von Boysen Jensen (1928, 1933) angegebene Methode gewählt. Die für die Versuche benutzte Luft wurde einer Stahlflasche entnommen, deren CO_2 -Gehalt bestimmt wurde und sich auf 0,656 mg CO_2 /L bei 0° , 760 mm belief. Da es unter Vorversuchen sich als schwierig erwies, die Spaltöffnungen während des Versuches geöffnet zu halten, musste die Flaschenluft durch 3 U-Rohre, versehen mit feuchtem Filtrierpapier, etwas Wasser und einem Tropfen Schwefelsäure, hindurch geleitet werden. Auch im Blattrezipienten erwies es sich als notwendig, unter dem Drahtnetz, worauf das Versuchsblatt ruhte, ein feuchtes Stück Filtrierpapier anzubringen. Als Absorptionsflüssigkeit für die Kohlensäure wurde eine n/20 Barytlösung verwendet. Nach dem Versuch wurde die Hauptmenge des Baryts durch Zusatz von genau 20 ml n/20 HCl neutralisiert und dann mit einer n/100 HCl zu Ende titriert. Jeder Versuch dauerte 6—7 Minuten und die stündlich durch den Apparat gesaugte Luftmenge betrug 17—19 Liter. Nach den Angaben von Boysen Jensen (1933) und Kjær (1937) werden in diesem Fall etwa 99 % der Kohlensäure im Barytwasser absorbiert.

Spaltöffnungen. Die Blätter von *Anemone nemorosa* sind heterobar, d.h. die Interzellularen kommunizieren nicht. Die Spaltöffnungen lassen sich besonders schön mittels des Stomatometers von Boysen Jensen (1928) kontrollieren. Vor jedem Photosyntheseversuch wurde an einem Parallelblatt die Öffnungsweite der Stomata mit diesem Stomatometer bestimmt, und

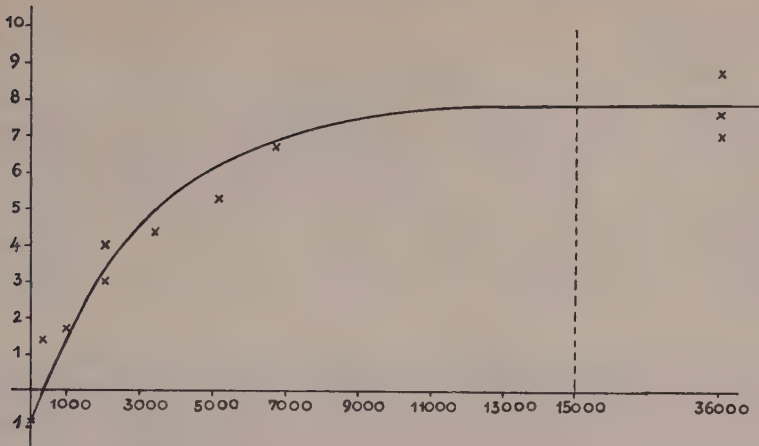


Abb. 1. *Anemone nemorosa*. Ordinate: mg CO₂ aufgenommen (oberhalb der Abscissenachse) oder ausgeschieden, beides pro 50 cm² Blattfläche, einseitig gemessen, pro Stunde bei 20° und einem CO₂-Druck von 0,228 mm. Abscisse: Beleuchtungsstärke in BJ-Lux.

gleich nach Abschluss des Versuches die Spaltöffnungsweite am Versuchsblatt selber konstatiert. Bei geöffneten Stomata war ein Druck von nur 20 cm Hg notwendig, um die Blätter bis zu 50 % mit Wasser zu infiltrieren; bei geschlossenen dagegen ein Druck von mehr als 20 cm Hg.

Lichtmessung. Die Lichtmessungen wurden mit einem Bercovitz Beleuchtungsmesser vorgenommen. Die Selen-Sperrschicht-Photozelle (empfindliche Fläche 25 cm²) wurde mit dem Farbglass von Schott u. Gen. OG 2 (1 mm dick) bedeckt und darüber ausserdem mit einer 1,5 mm dicken, auf der gegen die Lichtquelle wendenden Seite, mattierten Milchglasscheibe versehen. Auf diese Weise montiert, wurde der Beleuchtungsmesser auf der Photometerbank gegen einen sekundären Lichtstärke-Standard, Farbtemperatur 2848° K, geeicht. Dieser Standard war in Teddington, England, in candela geeicht worden, so dass die gemessenen Werte Luxwerte und nicht wie früher Hefnerluxwerte sind (1 Lux = 1,09 Hefnerlux). Da wir filtriertes Licht messen, werden die Einheiten, wie üblich, weiterhin als BJ-Lux benannt werden. Nur ist zu beachten, dass die hier gemessenen BJ-Lux nicht BJ-Hefnerlux sind.

Photosynthese bei *Anemone nemorosa*. Die maximale scheinbare Photosynthese von *Anemone nemorosa* beläuft sich im Mittel auf 7,9 mg CO₂ pro 50 cm² Blattfläche (einseitig) pro Stunde bei 20° und einem CO₂-Druck von 0,228 mm, 0,59 mg CO₂ pro Liter (siehe Abb. 1 u. Tabelle 1). Die reelle Photosynthese für *A. nemorosa* ist demnach 7,9 mg + 0,67 mg = 8,6 mg CO₂ pro 50 cm² Blattfläche (einseitig) pro Stunde. Die maximale apparente Photosynthese erreicht bei ausgesprochenen Lichtblättern von *Sinapis alba*

Tabelle 1. *Photosynthese der Blätter von Schatten- und Lichtpflanzen*. Alle Werte gelten pro 50 cm² Blattfläche, einseitig gemessen, pro Stunde bei 20° und einem CO₂-Druck von 0,228 mm.

Pflanze	Kompensationspunkt bei	Maximale apparente Photosynthese		
		mg CO ₂ aufgenommen	mg Trockensubstanz gebildet	erreicht bei Beleuchtungsstärke
Oxalis acetosella	100—200 BJ-Lux	0,8	0,5	1500 BJ-Lux
Anemone nemorosa ...	300 »	7,9	4,8	11000 »
Sinapis alba	500—700 »	14	8,5	15000 »

einen Wert von ungefähr 14 mg CO₂ (Müller 1932), bei extremen Schattenblättern von *Oxalis acetosella* nur einen von etwa 0,8 mg CO₂ pro 50 cm² Blattfläche (einseitig) pro Stunde bei 0,228 mm CO₂-Druck (Boysen Jensen 1918). *Anemone nemorosa* kommt also mit einem Wert von 7,9 mg CO₂ weder in die Kategorie der ausgesprochenen Schattenblätter noch in die der ausgesprochenen Lichtblätter. Der Wert der maximalen Photosynthese ist aber dem der Lichtblätter genähert. Die Licht-Photosynthesekurve von *A. nemorosa* ähnelt sehr der von *Cucumis sativus*, nach den Untersuchungen von Gabrielsen 1934.

Anemone nemorosa benötigt eine Beleuchtungsstärke von etwa 11000 BJ-Lux im Vergleich zu Lichtpflanzen mit etwa 15000 und Schattenpflanzen mit etwa nur 1500 BJ-Lux, um die maximale Photosynthese zu erreichen. Auch der Kompensationspunkt für *Anemone nemorosa*, belegen bei etwa 300 BJ-Lux, und die aus der scheinbaren Photosynthese berechnete gebildete Trockensubstanz zeigen diese Mittelstellung. Die Tabelle 1 wird dieses veranschaulichen.

Anemone nemorosa vermag wahrscheinlich nicht ausgesprochene Schattenblätter auszubilden; denn eine Blattprobe aus einem Bestand an der Lichtgrenze von *Anemone* (12 % des freien Tageslichtes vor der Laubentfaltung der Bäume) hatte eine maximale apparente Photosynthese von 7,1 mg CO₂ pro 50 cm² Blattfläche pro Stunde bei 20°, ein Wert, der nur wenig von dem der Anemonenblätter von helleren Standorten abweicht.

Respiration. Die Respirationsbestimmungen wurden alle nur an Blattflächen vorgenommen und mit der Luftstrommethode durchgeführt. 15 g Frischgewicht Blätter wurden in einem 300 ml Erlenmeyerkolben in einem Wasserbad, dessen Temperatur konstant gehalten wurde, angebracht. Ein kohlendioxid-freier Luftstrom führte unten in den Kolben hinein und oben wieder heraus. Das durch die Blätter ausgeschiedene CO₂ wurde vom Luftstrom aufgenommen und passierte zusammen mit diesem 30 cm³ n/20 Barytlösung, die das CO₂ absorbierte. Die Luftstromgeschwindigkeit betrug

Tabelle 2. *Atmung und Arealgewicht der Blätter von Schatten- und Lichtpflanzen.*

Pflanze	Temperatur	mg CO ₂ pro 50 cm ² Blattfläche (einseitig gemessen) pro Stunde	mg CO ₂ pro 1 g Frischgewicht pro Stunde	Areal von 1 g Blattfläche Frisch- gewicht in cm ²
<i>Oxalis acetosella</i> ... (Schattenpflanze)	20°	0,1	0,18	89
<i>Anemone nemorosa</i>	20°	0,67	0,89	67
	10°	0,20	0,27	
<i>Sinapis alba</i> (Lichtpflanze)	20°	0,8	0,77	48

12 Liter in der Stunde. Durch Titrierung von 25 cm³ der Barytlösung mit n/20 HCl konnte bestimmt werden, wieviel mg CO₂ in der Stunde pro 50 cm² (einseitig) ausgeschieden wurde. In Tabelle 2 wurden die Werte einer ausgesprochenen Schattenpflanze, *Oxalis acetosella*, und einer ausgesprochenen Lichtpflanze, *Sinapis alba*, zum Vergleich herangezogen. Die Mittelstellung von *Anemone nemorosa*, die aber mehr den Lichtpflanzen genähert ist, geht eindeutig daraus hervor.

Blattareal und Stoffmenge in Anemonen- und Oxalisbeständen

Anemone nemorosa. Am 18. Mai 1951 wurde aus einem etwa 100-jährigen Buchenbestand in Ermelunden, ungefähr 12 km nordwestlich von Kopenhagen, ein Areal von 0.80 m² in einer dichten Anemonenbewachung abgegrenzt. Alle oberirdischen Teile, die auf diesem Terrain wuchsen, wurden sorgfältig eingesammelt und das Gesamtfrischgewicht bestimmt zu 345 g mit einem Trockenstoffprozent von 16. An 15 g des Frischgewichtes konnte mit Hilfe des Ozalid-Lichtpausverfahrens das Blattareal ermittelt werden. 68,7 % = 237 g des gesamten Pflanzenmaterials ist Blattfläche, das übrige Stengel. 1 g Blattfläche hat ein Areal von 64,9 cm². Dieser Wert ist dem vergleichbaren von Lichtblättern sehr genähert. Aus den gefundenen Grössen wurde das oberirdische Frischgewicht zu 4,3 t, die oberirdische Trockensubstanz zu 0,7 t und das Blattareal zu 1,9 t pro ha Bodenflächeneinheit bestimmt. Die Zahlen sind mit Werten, die mir Prof. Müller überlassen hat, in Tabelle 3 verglichen.

Anemone nemorosa aus 140-jährigem Buchenwald (Geels Skov, Holte) in Nordost-Seeland. Dicht gedrängter Anemonenbestand, 7,888,000 Blumen pro ha, nur vereinzelte Pflanzen von *Melica uniflora*, *Viola silvatica* und Keimpflanzen von *Fagus silvatica*. Blattfläche am 20/4 1945 2,1 ha pro ha. 1 g Blattfläche Frischgewicht = 69 cm². Oberirdische Trockensubstanz 20/4 1945

Tabelle 3. *Stoffproduktion von Licht- und Schattenpflanzen.*

Pflanze	Oberirdische Trockensubstanz in t pro 1 ha Bodenfläche	Blattareal (einseitig gemessen) in ha pro 1 ha Bodenfläche	Beleuchtungsstärke in Prozent des freien Tageslichtes über dem Bestand
<i>Oxalis acetosella</i>	0,284	1,5	5
<i>Anemone nemorosa</i>	0,69	2,2	50
	0,83	2,1	55
	0,7	1,9	49
<i>Sinapis alba</i>	9,3	4,0	100
(Hornberger 1885)			

0,69 tons pro ha, hiervon 0,52 tons Blattspreiten. Beleuchtungsstärke über dem Bestände 50 % des vollen Tageslichtes.

Anemone nemorosa aus 140-jährigem Buchenwald (Geels Skov, Holte) in Nordost-Seeland. Dicht gedrängter Anemonenbestand, 7,510,000 Blumen pro ha, nur vereinzelte Pflanzen von *Asperula odorata*, *Oxalis acetosella* und Keimpflanzen von *Fagus silvatica*. Blattfläche am 27/4 1945 2,2 ha pro ha. 1 g Blattfläche Frischgewicht = 70 cm.² Oberirdische Trockensubstanz 27/4 1945 0,83 tons pro ha. Beleuchtungsstärke über dem Bestände 55 % des vollen Tageslichtes.

Oxalis acetosella. Dichter Bestand in der Peripherie eines 50-jährigen Fichtenwaldes (Rude Skov) in Nordost-Seeland. Ausser *Oxalis* vereinzelte Pflanzen von *Anemone nemorosa*. Blattfläche am 27/5 1946 1,5 ha pro ha. 1 g Blattfläche Frischgewicht = 89 cm.². Oberirdische Trockensubstanz 0,284 tons pro ha. Beleuchtungsstärke über dem Bestände 5 % des vollen Tageslichtes.

Zusammenfassung

- 1) Die maximale apparente Photosynthese von *Anemone nemorosa* beläuft sich im Mittel auf 7,9 mg CO₂ pro 50 cm² Blattfläche (einseitig) pro Stunde bei 20° und einem CO₂-Druck von 0,228 mm und der Kompensationspunkt ist ungefähr 300 BJ-Lux. Der gefundene Wert wurde mit Werten einer ausgesprochenen Schattenpflanze, *Oxalis acetosella*, und einer ausgesprochenen Lichtpflanze, *Sinapis alba*, verglichen.
- 2) Die Respirationsbestimmungen für *Anemone nemorosa* ergaben bei 20° 0,67 mg CO₂ und bei 10° 0,20 mg CO₂, beides pro 50 cm² Blattfläche (einseitig) pro Stunde. Auch hier wurden Werte von *Oxalis acetosella* und *Sinapis alba* zum Vergleich herangezogen.
- 3) Die Bestimmung der oberirdischen Trockensubstanz in den dichtesten *Anemone*-beständen in dänischen Buchenhochwäldern ergab 0,7—0,8 t

pro 1 ha Bodenfläche und das Blattareal (einseitig) hatte eine Grösse von 1,9—2,2 ha pro 1 ha Bodenfläche.

- 4) Aus den Resultaten der Punkte 1, 2 und 3 und den erwähnten Vergleichen lässt sich schliessen, dass *Anemone nemorosa* weder in die Kategorie der ausgesprochenen Schattenpflanzen noch in die der ausgesprochenen Lichtpflanzen zu stellen ist. *Anemone nemorosa* nimmt eine Stellung ein, die der der typischen Lichtpflanzen genähert ist.

Literatur

- Boysen Jensen, P.: Studies on the production of matter in light- and shadow-plants. — Bot. Tidsskr. 36: 219. 1918.
- Über neue Apparate zur Messung der Kohlensäureassimilation, der Respiration, der Öffnungsweite der Spaltöffnungen und der Beleuchtungsstärke. — Planta 6: 456. 1928.
- Über die Bestimmung der Assimilationsintensität. — Planta 21: 368. 1933.
- Erwiderung zu der Mitteilung von H. von Dückere: Über die Berechnung von Assimilationswerten nach Boysen Jensen. — Biochem. Zs. 295: 427. 1937.
- Daxer, H.: Über die Assimilationsökologie der Waldbodenflora. — Jb. wiss. Bot. 80: 363. 1934.
- Gabrielsen, E. K.: Untersuchungen über den Kohlenstoffhaushalt einer Gewächshauspflanze im Winter bei Tageslicht und mit elektrischer Zusatzbeleuchtung. — Planta 22: 180. 1934.
- Hornberger, R.: Untersuchungen über Gehalt und Zunahmen von *Sinapis alba* an Trockensubstanz u. chemischen Bestandteilen in 7-tägigen Vegetationsperioden. — Landw. Versuchsstat. 31: 415. 1885.
- Kjær, A.: Die Schwankungen der Assimilationsintensität der Blätter von *Sinapis alba* im Laufe des Tages in Abhängigkeit von inneren Faktoren. — Planta 26: 595. 1937.
- Lundegårdh, H.: Die Kohlensäureassimilation der Zuckerrübe. — Flora 121: 294. 1927.
- Klima und Boden in ihrer Wirkung auf das Pflanzenleben. — 3. Aufl., Jena 1949.
- Müller, D.: Analyse der verminderten Stoffproduktion bei Stickstoffmangel. — Planta 16: 1. 1932.
- Über Kohlensäureassimilation in normal und invers beleuchteten Blätter. — Planta 29: 215. 1938.
- u. P. Larsen: Analyse der Stoffproduktion bei Stickstoff- und Kalimangel. — Planta 23: 501. 1935.
- Romose, V.: Ökologische Untersuchungen über *Homalothecium sericeum*, seine Wachstumsperioden und seine Stoffproduktion. — Dansk Bot. Arkiv. 10, Heft 4, 1940.

On the Effect of Chlorate upon the Nitrate Reduction of Plants II. The Effect upon the Nitrate-reducing System in *Escherichia coli*

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Introduction

To obtain a clear picture of the effect of chlorate, it was felt desirable to study the enzymatic processes to which the effect seems to be connected, i.e. the reduction of nitrate to nitrite, more closely than could easily be done with *Aspergillus oryzae* (Goksøyr, 1951). Therefore, *Escherichia coli* was selected as a new test organism. *E. coli* has been shown to possess an enzyme system which is capable of reducing nitrate to nitrite in presence of suitable hydrogen donators (Quastel, Stephenson and Whetham, 1925, Stickland, 1931). There are also some reports about a further reduction to hydroxylamine and ammonia (Woods, 1938, Lascelles and Still, 1946). Suitable hydrogen donators are, e.g. gaseous hydrogen, formate, lactate, succinate, mannitol.

Detailed investigations regarding this system have been made by Stickland (1931) and by Yamagata (1938). Stickland used washed and aerated suspensions of the bacteria, in some experiments bacteria being treated with toluene. He found that the bacteria-suspension reduced nitrate to nitrite quantitatively, and at almost constant rate. The reduction was inhibited to 50 per cent by a cyanide concentration of 0.0001 *M*. Carbon monoxide did not inhibit the reduction (with lactate as hydrogen donator). Oxygen in-

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hibited the reduction strongly, i.e. 61 per cent in 1.09 per cent O_2 . — He concludes that nitrate reduction is due to another enzyme than the oxygen reduction, but these enzymes compete for the activated lactic acid. Yamagata was able to bring the nitrate reducing system into solution by autolysing bacteria which had been grown on a nitrate-lactate peptone medium. Her experiments give final evidence showing that the nitrate-reducing system consists of two parts: the dehydrogenase system and the nitrate reductase system. The H-transfer between these systems can be performed by a suitable redox-indicator, e.g. methylene blue.

Quastel, Stephenson and Whetham (1925) studied also the utilisation of *chlorate* as hydrogen acceptor by *E. coli*, and they found that this ion strongly oxidized lactate and succinate in presence of resting bacteria. In the substrate they could afterwards find *chlorite*, which had a toxic effect upon the bacteria.

Experimental technique

Cultural. — The experiments reported here have been performed with a stock of *E. coli* obtained from the University Biochemical Laboratory, Helsinki. The bacteria were cultivated in a Difco peptone broth, to which was normally added 0.5 per cent sodium lactate and 0.5 per cent sodium nitrate. After two days of growth at $37^\circ C$, the culture suspension was centrifugated, washed twice with distilled water and resuspended in a phosphate buffer of pH 7.5, which contained 0.5 per cent NaF. Normally, bacteria from two liters of culture were suspended in 100 ml buffer solution, as this gave a suitable reducing activity. As the dehydrogenase-part of the reducing system was not investigated, it was not considered necessary to aerate the bacteria suspension before the use.

Some experiments were made with autolysed bacteria. The method used is that described by Yamagata (1938), with the exception that the temperature during the autolysis was 37° , and the period 2–3 days. After the autolysis, the suspension was centrifugated twice for about 20 minutes at appr. 5000 r.p.m. The solution obtained was clear and yellowish, with a weak opalescence.

Experimental. — In most cases, ordinary Thunberg technique has been used. Thunberg tubes with a hollow, U-shaped stopper were used, and they were evacuated by means of a water pump. Experimental temperature has been 30° . Normally, the tubes were filled as follows: In the main body: 1 ml H_2O , 0.2 ml methylene blue 1 : 5000, 0.1 ml sodium lactate 1-M, 0.2 ml sodium nitrate (or chlorate). In the hollow stopper: 1 ml of the *coli* suspension. The reaction has always been stopped by addition of 0.5 ml 2-M NaOH, when more than a minute or so has lasted between the end of the experiment and before the start of the analysis of the contents of the tube.

Analytical. — The substances determined during the experiments are nitrite and chlorite.

Chlorite, was determined by titration with KJ and potassium thiosulfate, in about 1-M acetic acid. At the pH of 1-M acetic acid, about 2.5, it was found that no chlorate reacted with KJ, whereas the reaction with chlorite was complete within a

short time. It is important that the KJ is added before the acid, as else losses will occur due to the formation of chlorine dioxide. Starch was used as indicator, and 0.01 or 0.0025 *N* thiosulfate as titrating agent, according to the amount of chlorite present. Nitrite interferes however with this titration, as it reacts with KJ. This reaction is considerably slower and more incomplete than the reaction with chlorite. It was found that concentrations of nitrite below 0.0002-*M* did not interfere seriously, when the amount taken for titration was 1 ml. As the amount of chlorite in its turn must be restricted, when nitrite is to be determined, the error in the chlorite determinations will be about 10 per cent.

Nitrite, can be determined in different ways. In some preliminary experiments, which are omitted here, it was determined manometrically, by means of sulfamic acid, in the Warburg apparatus. This is a simple and convenient method for determining larger amounts of nitrite, and can easily be combined with measurements of gas exchange in the suspension.

In the experiments reported here, nitrite has been determined in the ordinary way, by means of sulphanilic acid and α -naphthylamine. Chlorite interferes with this determination. It was found that in presence of 0.0002-*M* chlorite, the extinction values on the Klett colorimeter were about 15 per cent below the values in absence of chlorite. There did not seem to be a very reproducible relationship between chlorite concentration and decrease in the Klett reading. However, the analytical error could be kept below 7–8 per cent by making a linear correction for the chlorite error, if the chlorite concentration was below 0.0004-*M*.

Consequently, when determining nitrite and chlorite in presence of each other by the methods described here, the concentrations in relation to each other must be kept within rather narrow limits. If one ml. aliquot is used for each analysis, the limits for nitrite are between 10 and 200 μM , and for chlorite between 25 and 400 μM per litre. This had to be taken into consideration when planning the experiments.

Finally, it may be added that the titration of chlorite and the colorimetric determination of nitrite were performed without removing protein or methylene blue from the solution, as this did not seem necessary.

Further experimental technique will be described in connection with actual experiments.

The reduction of chlorate

As already stated, Quastel *et al.* (1925) found *chlorite* as the reduction product, when a resting suspension of *E. coli* was treated with chlorate and lactate or succinate.

They also demonstrated that the reduction of chlorate was an enzymatic process.

Further evidence showing that the reduction product actually is chlorite, was found by the author by means of paper chromatography (figure 1). Recent papers demonstrate the use of that method also in detecting and estimating inorganic ions.

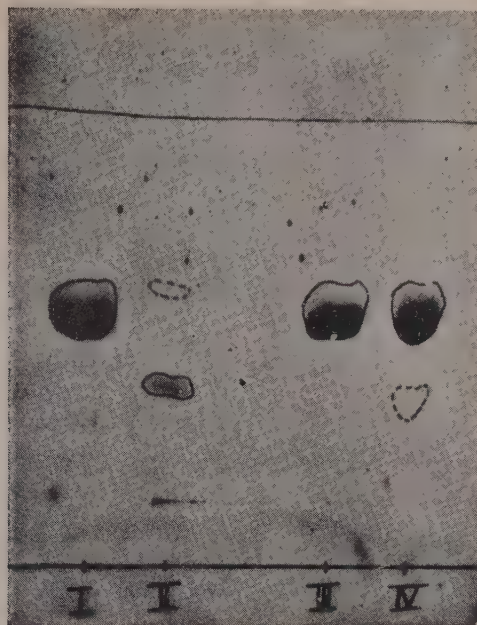


Figure 1. *Chromatographic detection of chlorite produced by reduction of chlorate by Esch. coli.* Spot I: 0.04-*M* NaClO_3 , II: 0.01-*M* NaClO_2 (the preparation was contaminated with chlorate), III: A coli suspension containing 0.04-*M* NaClO_3 , lactate and methylene blue, IV: The same suspension after 3 hours in the Thunberg tube. Titrated content of chlorite: 0.005-*M*. Size of the drops were 20 μl , the paper is Munktell 20/150. The liquid front has traveled 28 cm.

The solvent used is that proposed by Pollard, McOmie and Elbeih (1951), *viz.*: Butanol, pyridine and 1.5-*n* ammonia in the ratio 2 : 1 : 2. The solvent ascending technique was used. Satisfactory development of the spots was obtained by the use of a solution of aniline in HCl as described in »Organic Analytical Reagents», 1947. This gives a bluish green colour with chlorate, and with chlorite a spot which is violet for about half a minute, before turning bluish green. The colour with chlorite comes soon after the spraying, whereas the chlorate spot is not visible before the paper is practically dry. The reaction is sensitive; it was found that with 10 μl drops, concentrations of about 0.005-*M* can be detected. — With hypochlorite, chlorate spots only were found, probably as hypochlorite is too unstable to be chromatographed. Chloride and nitrate give no colour with this reagent, nitrite a reddish colour.

It should however be noted that the concentrations necessary for chromatographic detection, in this connection are abnormally high, so that the method could not be used generally in this work. Only in young and vigorously reducing suspensions could sufficiently high concentrations of chlorite be obtained. More normally, the reduction of chlorate stopped before the sus-

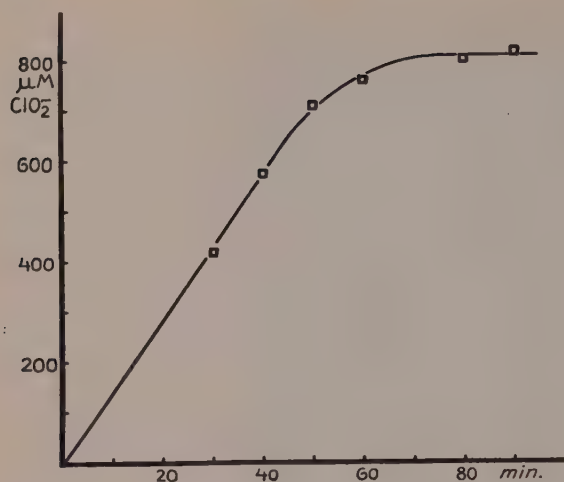


Figure 2. *Reduction of chlorate to chlorite. Time curve. Initial concentration of chlorate 800 μ M per litre.*

pension contained more than 0.003—0.004 moles of chlorite per litre (see later).

The order of reaction of the chlorate reduction was measured with Thunberg technique, six tubes being used. The result from one experiment will be seen in fig. 2.

In common with the nitrate reduction, it is seen here that the reduction proceeds with constant speed until very low concentrations of the substrate are left. Stickland (1931) made an approximate determination of the Michaelis constant for the nitrate reduction, and found values about $3-5 \cdot 10^{-5}$ at 45° . The low substrate concentrations made the determinations very insecure. The Michaelis constant for the chlorate reduction will be as insecure, but according to this and another experiment it must be somewhat larger, and probably lies around $8-10 \cdot 10^{-5}$.

Comparision of the reaction velocities of the nitrate and chlorate reductions, showed that the coli suspension reduced chlorate with a speed almost compatible with that of nitrate. This can be seen from table 1.

Table 1. *Reduction velocity of chlorate as compared with that of nitrate. Reaction time one hour. Concentrations given in μ moles per liter.*

Exp. no.	ClO_2^- v_1	NO_2^- v_2	$\frac{v_1 \cdot 100}{v_2}$
1	268	422	64
2	350	461	76
3	310	460	67
4	310	437	71

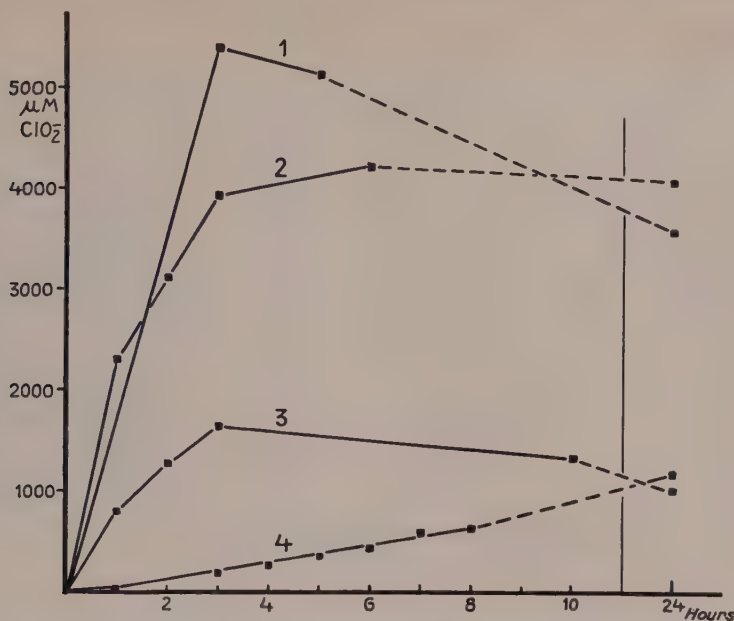


Figure 3. *Reduction of chlorate to chlorite. Maximal obtainable concentrations. Curve no. 1 made by Thunberg technique (at room temperature), the others by the burette technique.*

The effect of chlorite

A few experiments were made to study how far the reduction of chlorate would proceed, before the concentration of chlorite obtained, would stop the reaction. It was found that very great differences in the maximal obtainable concentrations of chlorite occurred with different coli suspensions. Some of these experiments were made according to a special technique, which made it easier to obtain time-curves of the reaction.

20 ml. of a coli suspension, together with 20 ml. H₂O, 4 ml. lactate, 4 ml. methylene blue and 2 ml. chlorate were mixed and filled in a burette. Out of this were taken with suitable time intervals samples of 1 ml, which were titrated according to the normal procedure. In the start the reduction will be slow, but when the oxygen dissolved in the mixture is used, the reaction will proceed with normal velocity. The diffusion of oxygen into the tube will only affect the uppermost layers, and be of no importance. The sedimentation velocity of a good coli suspension is also so slow that scarcely any error should occur due to it.

The experiments with this technique have all been carried out at room temperature.

Some of the results are compiled in fig. 3 and 4. In the experiments whose results are given in fig. 4, the ability of the suspension to reduce nitrate after

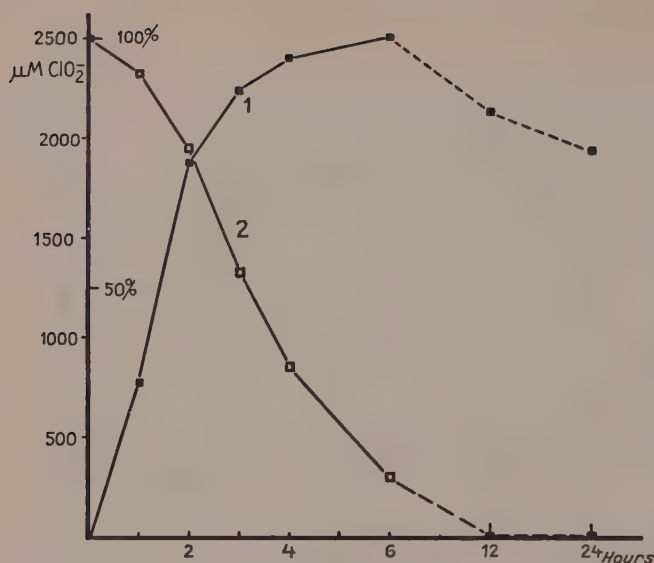


Figure 4. Reduction of chlorate to chlorite, and the effect upon the nitrate-reducing system. Curve 1: Chlorite production, measured by the burette method. Curve 2: The nitrate-reducing ability of samples taken out of the burette, washed and resuspended in buffer solution, measured by Thunberg technique.

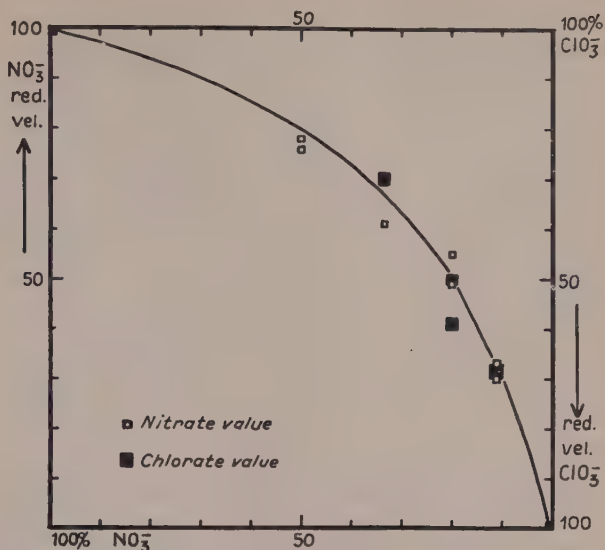
washing and resuspending was also investigated. It was observed that as the nitrate-reducing ability fell below 30 per cent, the colour of the methylene blue did not decrease during the experiment, showing that the dehydrogenase-part of the system was also inactive.

Quastel *et al.* (1925) found by growth experiments that chlorite concentrations as low as 0.005 per cent KClO_2 (about 470 μM per litre) seriously inhibited the multiplication of the bacteria. This is a concentration only 10 % of that which the organism itself is able to produce, according to these experiments. The action of chlorite upon the bacteria is probably of a more general character, but it needs some time to become manifest.

The interaction between the chlorate and nitrate reductions

So far, the reductions of chlorate and nitrate have been studied separately. If to a coli suspension a mixture of the two substances is added, it is found that an inhibition of the respective reductions occur. A number of experiments undertaken to study this in greater detail, demonstrated the competitive character of the inhibitions. It was also found that the percentage inhibitions of the two reactions always had a sum that approximated to 100, within the limits of error. In fig. 5 the results from three different experiments are compiled. In these experiments nitrate-chlorate mixture were used with a ratio varying

Figure 5. *The reduction velocities of nitrate and chlorate at different mixture ratios.* The nitrate reduction velocities are given from the bottom upwards, the chlorate reduction velocities from the top downwards. Velocities in a suspension containing only nitrate or chlorate given the value 100.



from 1 : 1 to 1 : 8, but with the sum of the concentrations of the two ions constant, as 0.08-*M* in the Thunberg tube.

To see whether the same relative inhibitions would occur with other concentrations, some experiments were made with a considerably lower concentration of the mixture, i.e. 0.008-*M*. In addition, some experiments were made with autolysed bacteria, to see whether this would make any difference. These results are compiled in table 2. The values given as »theoretical» in the table, refer to the curve drawn in fig. 5, and will be explained later.

The somewhat higher inhibition of the nitrate reduction, and smaller inhibition of the chlorate reduction, at the low concentration parallels with low nitrate content in the mixture, is explained by the fact that here the nitrate concentration is so small (about 890 μ *M*), that about 20 per cent of it will be removed during the experiment. This will relatively increase the amount of chlorate in the mixture. Otherwise a fairly good agreement is seen between the results, showing that the inhibitions are dependent on the nitrate/chlorate ratio only, and not upon the absolute concentrations. The inhibitions must thus be of a competitive character.

It can also be shown that the manner in which the inhibitions changes with the nitrate/chlorate ratio is in good agreement with that expected, if nitrate and chlorate compete for the same enzyme and combine with it to form intermediary compounds, the concentrations of which determine the velocities of the two reductions:

Table 2. *The inhibitions of the nitrate and chlorate reductions at different mixture ratios and total concentrations. Reaction time one hour, except for the autolysate experiment, where it was three hours. Concentrations given in moles per liter.*

	$\text{NO}_3^-/\text{ClO}_3^-$ ratio	Total concn. $\text{NO}_3^- + \text{ClO}_3^-$	ClO_3^- red. vel.	NO_3^- red. vel.	Sum
Suspension	1 : 4	0.008	54 %	48 %	102
»	1 : 8	»	83 »	25 »	108
»	1 : 8	»	81 »	29 »	110
Autolysate	1 : 4	0.08	—	61 »	—
»	1 : 4	»	—	58 »	—
»	1 : 8	»	—	30 »	—
» Theoretical »	1 : 1		20	80	100
»	1 : 2		33.3	66.7	100
»	1 : 4		50	50	100
»	1 : 8		66.7	33.3	100

Let c_1 mean the total concn. of substrate no. 1,

c_2 » » » » » no. 2,

E » » » » » the enzyme,

ES_1 and ES_2 the concns. of the enzyme-substrate complexes at equilibrium state.

We then have:

$$\frac{(E - \text{ES}_1 - \text{ES}_2) (c_1 - \text{ES}_1)}{\text{ES}_1} = k_1$$

$$\frac{(E - \text{ES}_1 - \text{ES}_2) (c_2 - \text{ES}_2)}{\text{ES}_2} = k_2$$

which, since ES_1 and ES_2 are very small compared with c_1 and c_2 , can be reduced to:

$$\frac{c_1}{c_2} \cdot \frac{\text{ES}_2}{\text{ES}_1} = \frac{k_1}{k_2}$$

If the velocity of reaction no. 1 is v_1 , and of reaction no. 2 is v_2 , we must have:

$$v_1 = m_1 \cdot \text{ES}_1$$

$$v_2 = m_2 \cdot \text{ES}_2$$

If the velocity of both reactions, when the other component is not present, is said to be, e.g. 100, we will have $m_1 = m_2$, and by further considering the sum of the concentrations of the two substrates constant, e.g. as 100, we will have:

$$\frac{v_1}{v_2} = \frac{k_2 c_1}{k_1 c_2}$$

and:

$$\frac{v_1}{100 - v_1} = \alpha \frac{c_1}{100 - c_1}; \quad \alpha = \frac{k_2}{k_1}$$

To find α , we have:

$$\alpha = \frac{\frac{v_1}{100 - v_1}}{\frac{c_1}{100 - c_1}}$$

We see from fig. 5 that in our case v_1 must be close to 50 when c_1 equals 20 (reaction no 1 : Nitrate reduction). That gives $\alpha=4$, and:

$$v_1 = \frac{400 c_1}{3 c_1 + 100}$$

which gives the fully drawn curve on fig. 5, and to which the »theoretical» values in table 2 refer.

It will also be seen that the constants k_1 and k_2 used here, actually are the Michaelis-constants of the two reactions. These must thus be expected to have a ratio of 1 : 4.

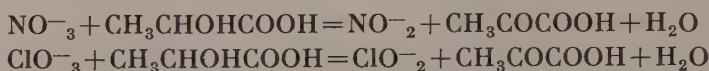
As mentioned earlier, the k_m value for the chlorate reduction has been estimated to $8-10 \cdot 10^{-5}$. Stickland found the value $3-5 \cdot 10^{-5}$ for the nitrate reduction, at 45° . Own attempts to determine it at lower temperature, seem to indicate that it must lie somewhere below $3 \cdot 10^{-5}$ at room temperature (the burette-method was used).

The results thus seem to prove that nitrate and chlorate actually are reduced by the same enzyme, the nitrate reductase in *E. coli*, and that they compete for this enzyme in accordance to the law of mass action.

It might be added that if the competition in this case was not for the enzyme surface, but for some velocity-restricting intermediate in the complex reaction, for instance leuco methylene blue, parallel to what Stickland (1931) found to be the case for the competition between nitrate and oxygen, another shape of the curve in fig. 5 should be expected. It should then have been S-shaped, and over a long interval there should have been no change of the inhibition with the nitrate/chlorate ratio, since the reductions of both at the concentrations here studied, proceed according to a zero order reaction.

Discussion

Thermodynamic considerations. To see which of the two reactions here in question, i.e.:



is energetically most preferable, we can study the reaction:



We can find the ΔF value for this reaction by means of the equation:

$$\Delta F = \Delta H - T\Delta S.$$

The heats of formation of the four ions are found in the compilation by Bichowsky and Rossini (1936). It should be noted that the Q_f value proposed for the chlorite ion is uncertain, as it is based on a value for HClO_2 , and complete dissociation of that acid is assumed. Better values for the chlorite ion are however not obtainable.

The entropies of the four ions are given in a collection by Latimer, Pitzer & Smith (1938).

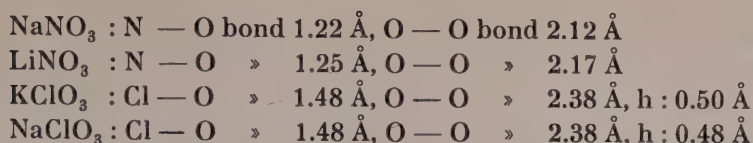
We have:

$$\begin{aligned}\Delta F &= -(Q_f)_{\text{NO}_3^-} - (Q_f)_{\text{ClO}_2^-} + (Q_f)_{\text{NO}_2^-} + (Q_f)_{\text{ClO}_3^-} \\ &\quad - T(S^\circ_{\text{NO}_3^-} + S^\circ_{\text{ClO}_2^-} - S^\circ_{\text{NO}_2^-} - S^\circ_{\text{ClO}_3^-}) \\ \Delta F &= -49190 - 13800 + 25300 + 20750 - 278 \cdot (35 + 24 - 29 - 39) \\ \Delta F &= -14440 \text{ cal at } 25^\circ \text{ C.}\end{aligned}$$

Thus, from an energetical point of view, the reduction of chlorate to chlorite is far more preferable than the reduction of nitrate to nitrite. Or, expressed another way, the oxidative potential of chlorate, when reduced to chlorite, is considerably greater than the oxidative potential of nitrate, when reduced to nitrite.

Structural considerations. In both reductions two electrons are involved, and one oxygen atom is removed from the substrate ion. It is remarkable that the reduction of chlorate stops at the chlorite level, as this is very difficultly obtained by ordinary reduction of chlorate; the reaction proceeds always to chloride (White, Taylor and Vincent, 1942). One is led to the conclusion that the enzyme must possess substrate specificity enough to stop the reduction of chlorate at the chlorite level, and also to distinguish between nitrate and chlorate in such a way as has been demonstrated by the experiments.

The structure of groups XO_3 in crystals has been investigated by Zacharia-sen (1931). He gives the following data for the nitrate and the chlorate ion:



Both ions are triangular, but the nitrate ion is planar, whereas the chlorate ion is slightly pyramidal (height = h). The reason for this difference is that the nitrate ion has 24 (3×8) valence electrons, whereas the chlorate ion has 26 ($3 \times 8 + 2$), the last two of which have maximal density in the fourth tetrahedral direction. They also give the chlorate ion a slight dipole moment, which the nitrate ion lacks.

Conclusive remarks. According to the experiments reported here, it lies near at hand to assume generally that the effect of chlorate — in full conformity with the theory by Åberg (1947) — is due to enzymatic reduction of chlorate to chlorite, which exerts its toxic effects upon the plant. Very little is known about the enzymes actually taking part in the nitrate reduction in plants, but a number of the nitrate-reducing enzymes known, show little substrate-specificity towards the hydrogen acceptor. This is e.g. the fact with the aldehyde oxidase in potato, and with the xanthine oxidase from milk. One might thus assume that nitrate-reducing enzymes will also be able to reduce chlorate — although, however, all enzymes may not have the same affinity ratio towards the two substances. Generally one should expect the inhibition by chlorate to have a competitive character. This is earlier found to be the case for higher plants like wheat, by a number of investigators (e.g. Åberg 1947), and recently by Fåhræus (1951) for the cellulose-decomposing bacteria *Cytophaga*. Apparently unique and in contradiction to other results are therefore the results found by the author (1951) with *Aspergillus oryzae*, which indicate a noncompetitive inhibition of the nitrate reduction in this organism. Future experiments will have to explain that discrepancy.

The different pictures of the chlorate poisoning may be explained by studying the sensitivity of the plants towards chlorite and its decomposition products, chlorine dioxide and hypochlorite.

Another conclusion which might be drawn from these experiments, is that in soil analysis for chlorate, methods should be used which allow a distinction between chlorate and chlorite, as by microbial action, the soil may contain a low, but fairly constant content of chlorite, as long as it contains chlorate.

Summary

1. *Escherichia coli* reduces chlorate to chlorite. The reduction proceeds with practically constant rate. The Michaelis-constant of the reduction is estimated to be $8-10 \cdot 10^{-5}$.

2. In presence of both nitrate and chlorate, the reduction of both substances is inhibited. The character of the inhibitions is investigated, and it is found that it agrees with the assumption that chlorate and nitrate are reduced by the same enzyme in the bacterial cell.
3. The nitrate and chlorate reductions are discussed energetically and structurally.
4. Conclusively it is found that the results support the theory of Åberg regarding the chlorate effect upon plants, and that the toxic effect of chlorate must be due to enzymatic reduction of this substance to chlorite.

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References

- Åberg, B.: On the mechanism of the toxic action of chlorate and some related substances upon young wheat plants. — *Lantbr.högs. annaler* 15: 1. 1947.
- Bichowsky, F. R. & Rossini, F. D.: The thermochemistry of the chemical substances. — N.Y., 1936: pp. 22, 32, 181.
- Fähræus, G.: Influence of nitrate concentration upon the chlorate toxicity in micro-organisms. — *Acta Chem. Scand.* 5: 1416. 1951.
- Goksøy, J.: On the effect of chlorate upon the nitrate reduction of plants I. Experiments with *Aspergillus oryzae*. — *Physiol. Plant.* 4: 498. 1951.
- Lascelles, J. & Still, J. L.: The reduction of nitrate, nitrite and hydroxylamine by *Escherichia coli*. — *Australian J. Exptl. Biol. Med. Sci.* 24: 159. 1946.
- Latimer, W. M., Pitzer, K. S. & Smith, W. V.: The entropies of aqueous ions. — *J. Am. Chem. Soc.* 60: 1829. 1938.
- Pollard, F. H., McOmie, J. F. W. & Elbeih, M.: The analysis of inorganic compounds by paper chromatography. Part II. Analysis of cations and anions. — *J. Chem. Soc.* 102: 470. 1951.
- Quastel, J. H., Stephensen, M. & Whetham, M. D.: Some reactions of resting bacteria in relation to anaerobic growth. — *Biochem. J.* 19: 304. 1925.
- Stickland, L. H.: Reduction of nitrates by *Bact. coli*. — *Biochem. J.* 25: 1543. 1931.
- Welcher, F. J.: *Organic Analytical Reagents* — N.Y. 1947, Vol II p. 267.
- White, J. F., Taylor, M. C. & Vincent, G. P.: Chemistry of chlorites. — *Ind. Eng. Chem.* 34: 782. 1942.
- Woods, D. D.: The reduction of nitrate to ammonia by *Closteridium Welchii*. — *Biochem. J.* 32: 2000. 1938.
- Yamagata, S.: Über die Nitratreduktase von *Bacterium coli*. Untersuchungen über die biologischen Reduktionen I. — *Acta Phytochim.* 10: 283. 1938.
- Zachariasen, W. H.: The structure of groups XO_3 in crystals. — *J. Am. Chem. Soc.* 53: 2123. 1931.

Inactivation of 3-Indoleacetic Acid by Peroxides

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There appears to exist a widespread belief that the auxin activity of 3-indoleacetic acid (IAA) is readily destroyed by peroxides. This view presumably stems from the observation of Nielsen (10) that extraction of aqueous preparations of rhizopin with ethyl ether caused complete loss of auxin activity unless the ether had been freshly distilled from ferrous hydroxide. It was assumed by Nielsen and also by Dolk and Thimann (1), who confirmed the observation, that peroxides in the ether were responsible. Rhizopin has been identified as 3-indoleacetic acid by Thimann (17).

A number of investigators have reported that addition of hydrogen peroxide to auxin preparations results in diminution of biological activity and several attempts have been made to utilize the presupposed peroxide lability for the separation and characterization of auxins, antiauxins, and auxin precursors in plant extracts (2, 3, 7, 8, 10, 18, 19).

The present experiments demonstrate that some, at least, of the observed effects of peroxides on IAA are not due to destruction of the latter and are reversible on removal of the peroxide.

Effect of Peroxides on Auxin Activity of IAA

In none of the above cited instances of diminution of biological activity by added peroxides does it appear to have been established that the peroxide was removed completely prior to the assay so that the possibility exists that

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Table 1. *Reversal of H₂O₂ inactivation of IAA.*

Mixture	Degrees curvature
IAA alone	19.3
IAA + H ₂ O ₂	3.9
IAA + H ₂ O ₂ + catalase	17.9

Nominal IAA concentration in test blocks = 50 µg./liter in all cases.

interference with the bioassay response rather than destruction of the auxin was responsible. The following experiments were performed in order to distinguish between these alternatives.

Hydrogen peroxide. — A solution containing 100 µg. IAA and 0.2 moles H₂O₂ per liter was allowed to stand at room temperature for 1 1/2 hrs. Catalase was then added to a portion of the solution and the mixture held for three-quarters of an hour longer, after which agar was added to both portions as well as to a comparably diluted sample of the IAA stock solution from which the original mixture had been prepared. Assay by the *Avena* curvature method showed (Table 1) that the inhibitory effect of H₂O₂ was almost completely prevented by incubation with catalase. It was established in preliminary tests that catalase, in the concentration here used, had no effect on the response to IAA and caused no curvature in the absence of IAA. Hence it appears that the loss of activity of IAA in the presence of H₂O₂ is due not to destruction of the auxin but to interference in the growth response.

The mode of interference by the peroxide is not clear. Concentrations of H₂O₂ which prevented completely the response to applied IAA did not alone reduce the elongation of *Avena* coleoptiles below that of the control when applied as in the straight growth test (20). It should be mentioned that both H₂O₂ (11) and benzoyl peroxide (14) have been claimed to act as auxins. We have been unable to confirm either of these observations; the activity of benzoyl peroxide has been questioned also by others (6).

Organic peroxides. — A similar reversal could be shown for the interference by the peroxides formed in ether. Evaporation at low temperature of a sample of ether (Merck purified, for fat extraction) yielded an aqueous residue amounting to about 1.5 % of the original volume. The peroxide content, as shown by titration with iodide, was equivalent to 1.0×10^{-3} millimoles per ml. of ether. IAA mixed with the residue was completely inactive by bioassay. As destruction of the organic peroxide by means of catalase was not feasible, recourse was had to a dilution technique. Aliquots of the residue were incubated with various amounts of IAA such that the

Table 2. *Reversal of inactivation of IAA by peroxides from ether.*

Composition of mixture		Dilution for bioassay	Degrees curvature
ml. of ether residue	µg. IAA		
0 ¹	0.1	1 : 2	18.1
1	0.1	1 : 2	0
1	1.0	1 : 20	6.4
1	10.0	1 : 200	19.1

¹ 1 ml. water used.

mixtures could be diluted 1 : 2, 1 : 20, or 1 : 200 for bioassay. The results (Table 2) show that the loss of activity was completely reversible if the inhibitory material was sufficiently diluted.

Effect of Peroxides on Color Reactions of IAA

Three principal chromogenic reagents, with numerous modifications, have been employed for the determination of IAA, (a) ferric ion in strong acid (13); (b) nitrite in acid solution (13); (c) cupric ion and glyoxylic acid in the presence of mineral acid (21).

The following experiments show that peroxides interfere with all these reactions.

Hydrogen peroxide. — For study of the metal ion-mineral acid type reagent, the Tang-Bonner (16) modification was chosen. The reagent was added to solutions containing IAA together with various amounts of H₂O₂

Table 3. *Effect of H₂O₂ on color tests for IAA.*

Concentration H ₂ O ₂ in IAA solution	Percent of initial IAA apparently destroyed		
	Tang-Bonner modification of Salkowski iron reagent ¹	Mitchell-Brunstetter modification of Salkowski nitrite reagent ²	Winkler-Petersen glyoxylic acid reagent ³
10 ⁻⁵ M	0		
10 ⁻⁴	24.1		6.7
10 ⁻³	56.0	6.3	57.9
10 ⁻²	83.6	21.4	91.7
10 ⁻¹		49.2	

¹ Test solution contained 29.0 µg. IAA per ml. Color measured after 20 min.² Test solution contained 100 µg. IAA per ml. Color measured after 2 hours.³ Test solution contained 62.5 µg. IAA per ml. Color measured after 4 hours.

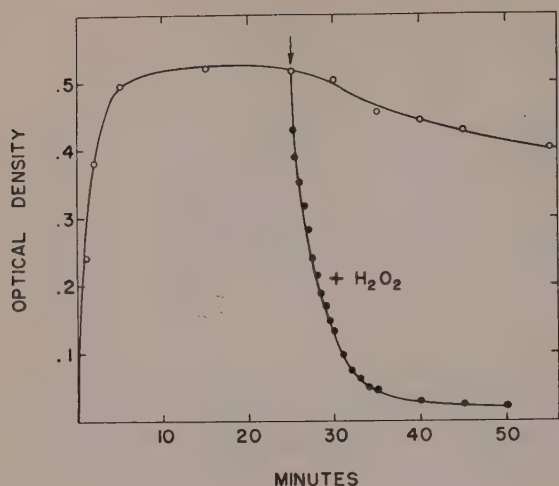


Fig. 1 — Effect of H_2O_2 on color produced by Tang-Bonner modification of Salkowski reagent for IAA. Open circles: — course of color development in absence H_2O_2 . Closed circles: — after addition 0.9 mmoles H_2O_2 to 10 ml. reaction mixture following maximum color development.

and the color densities measured after 20 minutes using an Evelyn photoelectric colorimeter with 540 $m\mu$ filter. As shown in Table 3, H_2O_2 at 10^{-4} M diminishes the color density by approximately one-fourth. Further tests showed that the apparent destruction occurred immediately upon addition of H_2O_2 to IAA and did not increase with time up to 60 minutes. The interference was independent of the pH of the H_2O_2 -IAA mixture in the range pH 1—9. Gordon and Weber (5) also have noted that H_2O_2 interferes in this reaction.

That the interference is not, however, due to destruction of IAA was shown by the following experiments. In the first place it was found that the intensity of the color produced by the reagent with IAA alone was decreased by addition of H_2O_2 after color development had reached its maximum as well as if added prior to treatment with the reagent (fig. 1). Secondly, if the peroxide was removed from the IAA- H_2O_2 mixtures before addition of the reagent there was virtually no loss of IAA. Removal of H_2O_2 could be accomplished either by reduction with arsenite or with catalase. Table 4 shows the result of an experiment by the first method. A similar result was obtained with catalase; the presence of catalase appears to intensify the color somewhat so that it is necessary to include catalase in the IAA standard solution.

Color development with both the Winkler-Petersen reagent and the nitrite reagent, of which the Mitchell-Brunstetter (9) modification was studied, also is interfered with by H_2O_2 (Table 3). In both cases the interference could be obviated by reduction of the peroxide with arsenite.

The intensity of the color produced by the Winkler-Petersen reagent is

Table 4. Recovery of IAA from mixtures containing H_2O_2 after reduction of the latter with arsenite.

Reaction mixture	IAA found ($\mu\text{g./ml.}$)
(1) IAA alone	31.5
(2) IAA + 0.9 mmoles ¹ H_2O_2	5.2
(3) IAA + 0.9 mmoles H_2O_2 ; then 1.5 mmoles Na_3AsO_3	30.5
(4) IAA + 1.5 mmoles Na_3AsO_3	32.2

¹ 1 mmole = 10^{-3} mole.

relatively stable for several hours. Addition of H_2O_2 during this period causes gradual fading. Interference of oxidants in this test for IAA has been noted previously whereas reducing agents are stated to be without effect (15).

An interesting application of the color test for IAA to the measurement of dosage of ionizing radiation has been suggested by Gordon *et al.* (4) who found that the apparent destruction of IAA in very dilute aqueous solution was proportional to the integral dose of radiation energy. The foregoing results suggest the possibility that the mechanism of this effect may in fact depend upon interference by H_2O_2 produced from the water.

Organic peroxides. — Indirect evidence has been presented (12) indicating that autoxidation of ethyl ether gives rise to only one peroxide, although the identity of this compound is still not established. The influence of the ether peroxide on colorimetric determination of IAA was studied by using an aqueous residue prepared by evaporation as described in a preceding section. Mixture of a 50 ppm IAA solution with an equal volume of this residue, containing 0.064 mmoles peroxide per ml., resulted in an apparent destruction of 24 % of the IAA as determined with the Tang-Bonner reagent. The ether-water partition coefficient of this peroxide is approximately 1, so that it is possible to separate the peroxide from the IAA in such a mixture by adjusting to an alkaline pH and extracting repeatedly with ether. After removal of the peroxide in this manner, 98 % of the original IAA was recoverable, again demonstrating that the interference does not involve destruction of IAA.

A similar result was found with *tert*-butyl hydroperoxide. In this case the peroxide was removed by reduction with arsenite. Employing the above described extraction procedure, quantitative recoveries of IAA were obtained also from mixtures with the following: di-*tert*-butyl peroxide, lauroyl peroxide, benzoyl peroxide, and *p*-menthane peroxide.

Discussion

These results show that the available methods for determination of small quantities of IAA, both colorimetric and bioassay, are subject to considerable interference by H_2O_2 and organic peroxides. Results of IAA determinations in the presence of peroxides may be quite misleading unless appropriate controls are included. It is suggested that conclusions as to the identity of various auxins, auxin precursors, antiauxins, and inhibitors which have been based on studies of peroxide sensitivity may require reexamination.

Summary

Hydrogen peroxide and a number of organic peroxides interfere in the determination of 3-indoleacetic acid both by the *Avena* bioassay and by the commonly used color reactions. The interference is not due to destruction of the indoleacetic acid and is reversible on removal of the peroxide.

We are indebted to Mr. James A. Lockhart for performing certain of the bioassays.

References

1. Dolk, H. E. & Thimann, K. V.: Studies on the growth hormone of plants. I. — Proc. Nat. Acad. Sci. 18: 30. 1932.
2. Funke, H.: Über Hemm- und Wuchsstoffe des keimenden Maiskorns. — Jahrb. wiss. Bot. 91: 54. 1943.
3. Funke, H. & Söding, H.: Über das Wuchsstoff-Hemmstoffsystem der Haferkoleoptile und der Kartoffelknolle. — Planta 36: 341. 1948.
4. Gordon, S. A., Stutz, R. E. & Weber, R. P.: Chemical dosimetry. — Argonne National Laboratory Quarterly Rept., Divn. Biol. & Med. Res., May-July 1950, p. 65.
5. — & Weber, R. P.: Colorimetric estimation of indoleacetic acid. — Plant Physiol. 26: 192. 1951.
6. Koepfli, J. B., Thimann, K. V. & Went, F. W.: Phytohormones: structure and physiological activity. I. — J. Biol. Chem. 122: 763. 1938.
7. Laibach, F. & Maschmann, E.: Über den Wuchsstoff der Orchideenpollinien. — Jahrb. wiss. Bot. 78: 399. 1933.
8. Meyer, E.: Über den Wuchsstoff-Hemmstoffgehalt gesunder und abbaukranker Kartoffelknollen. — Planta 38: 213. 1950.
9. Mitchell, J. W. & Brunstetter, B. C.: Colorimetric methods for the quantitative estimation of indole-3-acetic acid. — Bot. Gaz. 100: 802. 1939.
10. Nielsen, N.: Untersuchungen über einen neuen Wachstumsregulierenden Stoff: Rhizopin. — Jahrb. wiss. Bot. 73: 125. 1930.
11. Raadts, E.: Über den Einfluss der Ascorbinsäure auf die Auxinaktivierung. — Planta 36: 103. 1948.
12. Reimers, F.: Æter til Narkose. — Einar Munksgaards Forlag, Copenhagen. 1943.

13. Salkowski, E.: Über das Verhalten der Skatolcarbonsäure in Organismus. — Z. physiol. Chem. 9: 23. 1885.
14. Snow, R.: Two new chemical plant growth substances. — Nature 139: 27. 1937.
15. Sutter, E.: Die chemische Bestimmung des Heteroauxins und Versuche über seine Aufnahme durch die Pflanze. — Ber. schweiz. bot. Ges. 54: 197. 1944.
16. Tang, Y. W. & Bonner, J.: The enzymatic inactivation of indoleacetic acid. I. Some characteristics of the enzyme contained in pea seedlings. — Arch. Biochem. 13: 11. 1947.
17. Thimann, K. V.: On the plant growth hormone produced by *Rhizopus suinus*. — J. Biol. Chem. 109: 279. 1935.
18. von Guttenberg, H. & Lehle-Joerges, E.: Über das Vorkommen von Auxin und Heteroauxin in ruhenden und keimenden Samen. — Planta 35: 281. 1947.
19. Voss, H.: Nachweis des inaktiven Wuchsstoffes, eines Wuchsstoffantagonisten und deren wachstumsregulatorische Bedeutung. — Planta 30: 252. 1939.
20. Weintraub, R. L.: An assay method for growth-promoting substances utilizing straight growth of the *Avena* Coleoptile. — Smithsonian Misc. Coll. 97: no. 11. 1938.
21. Winkler, S. & Petersen, S.: Tryptophanreaktion und Nachweis des Heteroauxins. — Z. physiol. Chem. 231: 210. 1935.

Observations on the 'Staling' of White's Medium by Excised Tomato Roots. II. Iron Availability

By

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It has been shown that the continued growth of excised tomato roots in White's medium (White, 1943) causes a rapid reduction in its growth-promoting activity and evidence has been advanced that this cannot be entirely accounted for by the accumulation of monosaccharides and the direct effect of the more alkaline pH which develops (Street, McGonagle and Lowe, 1951). It seemed, therefore, that the 'staling' of the medium must also involve one or more of the following changes: (i) depletion of essential nutrients; (ii) changes in the relative proportions of the components of the medium; (iii) accumulation in the medium of toxic substances. The experiments described in this paper have shown that the alkaline pH drift renders the medium iron-deficient and that this is one of the major factors involved in 'staling'. Media have been prepared in which iron remains available despite the pH drift and have enabled us to make new observations on the growth rates of excised tomato roots and to study the mechanism by which they absorb iron.

Experimental

Culture technique

The general culture techniques, composition of the standard medium, planning and replication of experimental treatments and methods of measuring growth have been previously described (Street, McGonagle and Lowe, 1951). 100 ml wide-mouthed conical flasks containing 50 ml medium were used in all experiments. Except where

otherwise stated, each flask was inoculated with a single 10 mm root tip of a clone of excised tomato roots (Best-of-All) and was incubated at $27 \pm 0.5^\circ \text{C}$. The standard White's medium used contained added copper and molybdenum (cf. Boll and Street, 1951).

I. The stimulatory effect of iron added to 'staled' medium

Batches of White's medium in which excised tomato roots (1 root per 50 ml) had already been cultured for various periods were used in these experiments. These are referred to as 'staled' media, the period of 'staling' being indicated e.g. »7-day 'staled' medium». Such 'staled' medium was clarified by filtration through a No. 3 porosity Pyrex glass filter, readjusted to its initial pH with dilute »Analar» grade hydrochloric acid and then sterilised by filtration through a No. 5 porosity Pyrex glass filter. The experiments have shown the effects of adding various components of the medium upon the growth-promoting activity of the 'staled' medium. Root tips 10 mm in length were used as inocula in assaying such activity. It has been established that sucrose is not markedly depleted by the roots during 14 days' culture (Dormer and Street, 1949) and additions of sucrose to

Table 1. Alteration of the growth-promoting activity of new and 'staled' White's medium resulting from the addition of (i) a second 100 ml/l of the solution of inorganic salts (as White, 1943, but with additions to give 0.005 p.p.m. Cu and 0.001 p.p.m. Mo in final medium) or (ii) a second 2.5 mg/l Ferric sulphate ($\text{Fe}_2(\text{SO}_4)_3$). Media inoculated with 10 mm excised tomato root tips and incubated at $27 \pm 0.5^\circ \text{C}$. for 7 days.

Experiment No.	Medium	Increase in length of main (mm)	No. of laterals per root	Length of laterals per root (mm)	pH values	
					initial	final
1.	New standard medium	78.6	32.8	240	4.30	5.53
	— — — + 100 ml/l inorganic salt soln.	71.4	28.0	107	4.40	5.23
	7-day 'staled' medium (a)	53.6	17.8	84	4.24	4.45
	— — — + 100 ml/l inorganic salt soln.	28.2	7.5	20	4.20	4.43
	— — — + 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$	67.9	28.9	242	4.14	4.54
	14-day 'staled' medium (b)	24.7	7.7	21	4.36	4.40
	— — — + 100 ml/l inorganic salt soln.	2.8	1.0	1.0	4.30	4.29
2.	— — — + 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$	63.1	27.8	143	4.30	4.61
	New standard medium	82.2	30.1	242	4.39	5.53
	— — — + 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$	82.5	30.8	261	4.16	5.18
	14-day 'staled' medium (b)	24.6	7.4	14.5	4.33	4.49
	— — — + 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$	57.6	24.8	96	4.19	4.46

'Staled' medium: each 50 ml medium had supported growth of a single root tip for 7 days (a) or 14 days (b) at $27 \pm 0.5^\circ \text{C}$.

'staled' medium decreased its growth-promoting activity and produced, in the roots, symptoms associated with high sucrose concentrations (Street and McGregor, 1952). Addition of the vitamins of White's medium to the 'staled' medium had no effect upon its activity. Addition of the 'solution of inorganic salts', which includes all the mineral nutrients except ferric sulphate, markedly reduced the activity of the 'staled' medium. Doubling the salt concentration in new standard medium caused a reduction in growth, particularly in length of lateral roots and this deleterious effect of an additional supply of salts was intensified with the 'staled' medium (Experiment No. 1, Table 1).

White's medium contains 2.5 mg ferric sulphate per litre and doubling this concentration has no effect upon the root growth made in 7 days. However, when 2.5 mg/l ferric sulphate was added to 'staled' medium after pH adjustment but prior to filter-sterilisation, its growth-promoting activity was greatly increased. With 7-day 'staled' medium this addition made its growth-promoting activity almost equal to that of new medium and only in the growth in length of the main axis was a lower value recorded (Experiments Nos. 1 and 2, Table 1).

II. Evidence that the alkaline pH drift in White's medium leads to iron precipitation

Ferric and ferrous salts are readily soluble in acid solution but with increase in hydroxyl ion concentration their solubility decreases. With solutions exposed to air, the solubility is controlled by the equilibrium ratio of Fe^{2+} to Fe^{3+} and the solubility products at 18°C . are 1.1×10^{-36} for ferric hydroxide and 1.64×10^{-14} for ferrous hydroxide. Halvorson and Starkey (1927) give the following values for solutions in equilibrium with atmospheric oxygen and in contact with solid $\text{Fe}(\text{OH})_3$:

pH	Iron in solution in p.p.m.	
	Fe^{2+}	Fe^{3+}
3.0	2.7×10^3	6.1×10^1
4.0	2.7×10^0	6.1×10^{-2}
5.0	2.7×10^{-3}	6.1×10^{-5}
6.0	2.7×10^{-6}	6.1×10^{-8}
7.0	2.7×10^{-9}	6.1×10^{-11}

It is, therefore, to be expected that when iron is supplied as an inorganic salt in a medium devoid of constituents capable of forming stable iron complexes, little of the iron will be retained in solution at pH values of 5.0 and above. Iron chlorosis has frequently been reported in water and sand cultures, using solutions of neutral or alkaline pH with iron supplied as an

Table 2. *Effect on the growth of excised tomato root tips of the quantity of ferric sulphate included in White's medium and in this medium modified by supplying the standard nitrate-N concentration as ammonium nitrate (Exp. No. 3).*

Period of culture (days)	Medium	Increase in length of main axis (mm)	No. of laterals per root	Dry weight per root (mg)	pH values	
					initial	final
7	Standard medium.....	73.8	25.5	2.2	4.31	5.88
	— — — + 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$	62.6	20.3	1.6	4.18	4.98
	Medium containing NH_4NO_3	56.4	18.0	1.3	4.38	3.96
	— — — + 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$	45.6	20.0	1.0	4.15	3.85
14	Standard medium	85.6	30.2	4.7	4.36	6.44
	— — — + 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$	76.6	24.8	4.7	4.17	6.33
	— — — + 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$	91.8	33.9	5.0	4.31	6.32
	added on day 7 of culture ¹					
	Medium containing NH_4NO_3	82.1	25.3	8.0	4.10	4.02
	— — — + 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$	72.7	27.0	6.9	4.07	4.06
	— — — + 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$	85.0	26.2	7.1	4.36	3.96
	added on day 7 of culture ¹					

¹ added aseptically in the form of 5 ml sterile solution of ferric sulphate per 50 ml culture.

inorganic salt. Jones and Shive (1921, 1923) have noted that addition of ammonium sulphate to culture solutions containing either ferric phosphate or ferrous sulphate increases iron availability by keeping the pH acid. White's medium modified by substituting calcium and potassium chlorides for the corresponding nitrates and supplying nitrogen as ammonium nitrate to give the standard concentration of nitrate-N, will support the growth of excised tomato roots and does not show the alkaline pH drift characteristic of the standard medium (Street, McGonagle and Lowe, 1951). Comparison of the growth of excised tomato roots in this medium with that in the standard medium under conditions of modified ferric sulphate concentration has shown that (i) growth is not limited by the amount of ferric sulphate in White's medium provided iron precipitation is prevented by pH stabilisation; (ii) increase in the initial ferric sulphate concentration fails to increase iron availability in medium which becomes alkaline as a result of root growth (Experiment No 3, Table 2).

III. Growth of excised tomato roots in media containing various iron compounds

In preliminary experiments, various iron sources were tested at concentrations equivalent in iron content to the standard 2.5 mg/l ferric sulphate.

Table 3. *Growth of excised tomato roots in White's medium containing various iron sources at concentrations equivalent in iron content to 2.5 mg/l $\text{Fe}_2(\text{SO}_4)_3$ (Exp. No. 4).*

Period of culture (days)	Source of iron	Increase in length of main axis (mm)	No. of laterals per root	Length of laterals per root (mm)	Dry weight per root (mg)	pH values	
						initial	final
7	Ferric sulphate (Laboratory reagent quality) $\text{Fe}_2(\text{SO}_4)_3$	93.5	34.8	169	1.93	4.39	5.82
	Ferrous sulphate; 'Analar' $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$..	92.7	34.3	160	2.24	4.49	6.02
	Ferric ammonium sulphate 'Analar' $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$	92.3	36.0	233	2.20	4.40	5.81
	Ferric citrate — 'commercial scales'	89.0	32.0	185	2.07	4.85	6.13
	Ferric potassium tartrate — 'commercial scales'	78.0	30.0	146	1.85	4.75	5.90
14	Ferric sulphate	106.5	40.8		4.31	4.39	6.28
	Ferrous sulphate	99.0	38.0		4.19	4.49	6.31
	Ferric ammonium sulphate	100.3	36.0		4.97	4.40	6.38
	Ferric citrate	100.0	38.0		5.11	4.85	6.41
	Ferric potassium tartrate	85.0	33.0		3.62	4.72	6.32

Results of one such experiment (Experiment No. 4) are given in Table 3. All the iron sources, with the exception of ferric potassium tartrate, supported a high rate of growth. Ferrous and ferric sulphates gave very similar results probably due to the rapid establishment of the $\text{Fe}^{2+} : \text{Fe}^{3+}$ equilibrium ratio during autoclaving. The value of ferric citrate as a source of iron at neutral and alkaline pH values has been stressed by several workers (Hoagland, 1919; Tottingham and Rankin, 1923; Hopkins and Wann, 1926; Thomas, 1929; Clark, 1936).

A similar type of iron complex (ferric tartrate at 1.5 mg/l) was used by Bonner and Addicott (1937) in their work on the culture of excised pea roots and was subsequently included in the medium for the culture of roots of tomato and other species (Bonner and Devirian, 1939; Bonner, 1940). Its suitability as an iron source was tested, however, at a time when the medium in use was otherwise unsatisfactory and only capable of supporting a low rate of growth. In our preliminary experiments, excellent growth was obtained with ferric citrate. This compound gave the highest increases in dry weight between day 7 and day 14 of the growth period (the time of high pH). It was decided, therefore, to examine the possibility of using iron citrate at appropriate concentrations to overcome the check in growth which results from the alkaline pH drift.

IV. *Ferric citrate as an iron source for excised tomato roots*

It has generally been observed that high ferric citrate concentrations are required to maintain iron availability at high pH values although such concentrations may be toxic at a more acid pH (Hopkins and Wann, 1926; Clark, 1936; Bitcover and Sieling, 1951). An attempt was, therefore, made to find a concentration of commercial ferric citrate scales which would promote a high initial growth of excised tomato roots and maintain it as the pH became more alkaline. The results of Experiment No. 5 (presented in Table 4) can be summarised as follows: — During a 7-day culture period, ferric citrate at all concentrations gave no significant increase in growth over that of the control ($\text{Fe}_2(\text{SO}_4)_3$ at 2.5 mg/l). Concentrations of $\times 5$ and $\times 10$ the standard iron concentration clearly decreased the growth rate measured over this period. With a 14-day culture period, significantly higher values were obtained for dry weight per root at concentrations of $\times 1$ and $\times 2$ ferric citrate, and for increase in length of the main axis and number of laterals per root at $\times 2$ ferric citrate. The unfavourable effect of higher citrate concentrations was still in evidence. Thus it is clear that appropriate concentrations of ferric citrate can exert a beneficial effect when root growth is occurring at high pH values.

In one experiment on the effect of ferric citrate concentrations, the copper and molybdenum now regularly included in our standard medium (Boll and Street, 1951) were inadvertently omitted. This deficiency coincided with a change in the relative effects of the $\times 1$ and $\times 5$ concentrations of ferric citrate. After 14 days' culture the following mean values were obtained: —

Ferric citrate concentration	Increase in length of main axis (mm)	No. of laterals per root	Dry weight per root (mg)
$\times 1.0$	84.8	31.9	4.46
$\times 5.0$	106.1	38.5	8.30

This effect was also obtained when only the copper was omitted (Experiment No. 6, Table 5). These results suggested that the effect of $\times 5$ and $\times 10$ concentrations of the commercial sample of ferric citrate scales was at least partly due to copper contamination, and made desirable the preparation of a less-heavily-contaminated sample of the complex. This was obtained by using a spectrographically standardised ferric chloride solution (Johnson Matthey and Co., London, E.C. 1), 'Analar' grade citric acid and 'Analar' grade sodium hydroxide. Since Langford and Quinan (1948) have shown that the formation of the ferric citrate complex can be represented: —

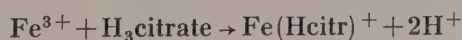


Table 4. *Effect of the concentration of ferric citrate 'commercial scales' on the growth of excised tomato root tips in White's medium (Exp. No. 5).*

Period of culture	Iron source	Iron concentration relative to standard	Increase in length of main axis (mm)	No. of laterals per root	Dry weight per root (mg)	pH values	
						initial	final
7 days	Ferric sulphate	× 1.0	96.4	30.8	2.19	4.38	5.47
	Ferric citrate	× 0.5	89.2	30.3	1.98	4.58	5.13
	» »	× 1.0	98.1	35.9	2.46	4.39	5.83
	» »	× 2.0	97.8	33.6	2.24	4.45	6.09
	» »	× 5.0	177.7	25.1	1.58	4.60	5.38
	» »	× 10.0	60.1	16.7	0.71	4.66	5.02
14 days	Ferric sulphate	× 1.0	127.2	40.9	4.43		6.11
	Ferric citrate	× 0.5	105.6	38.7	4.89		5.70
	» »	× 1.0	112.6	39.4	5.42		6.90
	» »	× 2.0	150.6	56.2	8.42		6.43
	» »	× 5.0	90.5	26.2	4.55		6.73
	» »	× 10.0	73.4	19.7	3.72		5.99

Table 5. *Effect on the growth of excised tomato root tips of various iron concentrations using ferric citrate 'commercial scales' in the absence of the 0.005 p.p.m. Cu normally included in the standard medium and using 'Ferric citrate solution 1:1' in the standard medium containing added Cu.*

Source of iron	Period of culture (days)	Iron concentration relative to standard	Increase in length of main axis (mm)	No. of laterals per root	Dry weight per root (mg)	pH values	
						initial	final
Ferric citrate 'commercial scales' (Exp. No. 6)	7	× 1	88.9	33.4	2.01	4.48	5.60
		× 5	86.4	29.1	2.07	4.67	5.98
		× 10	67.7	15.1	1.51	4.88	5.27
	14	× 1	113.1	37.0	5.20		6.83
		× 5	126.4	40.0	7.10		7.06
		× 10	122.2	33.5	6.85		6.93
'Ferric citrate solution 1:1' (Exp. No. 7)	7	× 1	77.9	31.3	1.82	4.63	5.88
		× 5	91.9	35.6	1.85	4.70	5.95
	14	× 1	122.0	41.9	5.1		6.64
		× 5	126.2	41.8	7.6		6.98

the ferric chloride and citric acid solutions were mixed in equimolar proportions. The pH was then brought to 4.6 by the addition of a solution of 'Analar' grade sodium hydroxide. This preparation was called 'Ferric citrate solution 1:1' and was adjusted in volume so that 1 ml was equivalent in iron content to 2.5 mg $\text{Fe}_2(\text{SO}_4)_3$. When this solution was used to give ×1

Table 6. *Growth of excised tomato roots in White's medium compared with that in media containing ferric citrate (Exp. No. 8).*

Feature measured	Iron source and concentration	Period of culture (days)							
		0	2	4	7	11	14	21	30
Increase in length of main axis (mm)	$\text{Fe}_2(\text{SO}_4)_3 \cdot \text{Fe} \times 1$		20.0	45.6	96.4	127.4	127.2	—	—
	Ferric citrate $\text{Fe} \times 2$		13.3	50.3	97.8	137.4	150.6	—	165.6
	» » $\text{Fe} \times 5$		—	35.6	76.1	117.1	134.7	164.6	158.2
No. of primary laterals per root	$\text{Fe}_2(\text{SO}_4)_3 \cdot \text{Fe} \times 1$		0.75	8.1	30.8	43.0	41.0	—	—
	Ferric citrate $\text{Fe} \times 2$		0.6	7.3	33.6	46.7	56.2	—	—
	» » $\text{Fe} \times 5$		—	3.9	23.2	33.8	39.2	—	—
Length of laterals per root (mm)	$\text{Fe}_2(\text{SO}_4)_3 \cdot \text{Fe} \times 1$		1.3	18.6	180.5	293	—	—	—
	Ferric citrate $\text{Fe} \times 2$		2.1	18.7	211.3	488	—	—	—
	» » $\text{Fe} \times 5$		—	7.3	118.3	455	—	—	—
Dry weight per root (mg)	$\text{Fe}_2(\text{SO}_4)_3 \cdot \text{Fe} \times 1$		—	—	2.19	4.45	4.47	5.07	—
	Ferric citrate $\text{Fe} \times 2$		—	—	2.24	5.21	8.42	15.9	22.1
	» » $\text{Fe} \times 5$		—	—	1.60	4.00	6.9	13.1	28.44
pH of culture medium	$\text{Fe}_2(\text{SO}_4)_3 \cdot \text{Fe} \times 1$	4.45	4.48	4.44	5.47	6.09	6.11	6.19	—
	Ferric citrate $\text{Fe} \times 2$	4.51	4.52	4.81	5.66	6.09	6.43	6.77	7.30
	» » $\text{Fe} \times 5$	4.53	—	4.53	4.86	5.83	6.22	6.64	6.80

and $\times 5$ the standard iron concentration in medium containing the standard copper addition, results were obtained which were only achieved with the commercial scale preparation when copper had been omitted (Experiment No. 7, Table 5). Therefore, 'Ferric citrate solution 1 : 1' was a more suitable source of the complex when higher iron concentrations were being tested.

Using the commercial scales at $\times 2$ standard iron concentration and the 'Ferric citrate solution 1 : 1' at the $\times 5$ concentration, growth measurements extending up to 30 days of culture were made (Experiment No. 8) and compared with those in the standard culture medium containing ferric sulphate at $\times 1$ iron concentration. The results, which are shown in Table 6, are summarised below.

(i) Both White's medium and the medium containing ferric citrate at $\times 2$ standard iron concentration supported a similar high rate of growth during the first 7 days of culture. The medium containing ferric citrate gave a higher value only for the length of laterals per root, which depends almost entirely on growth after the 4th day of culture.

(ii) Extension of the growth period beyond 7 days showed that supplying iron as ferric citrate enables growth to proceed for a longer time. In the medium containing $\times 5$ standard iron concentration, growth was initially much slower than in either of the other media and only from 14 days onwards was the beneficial effect of higher citrate concentration apparent. In White's

medium growth had virtually ceased by the 11th day of culture. In the media containing ferric citrate, growth was still proceeding after 30 days though it is clear that the increases in dry weight after 14 days are mainly due to lateral root growth. Growth of individual lateral roots must be severely checked by the end of the culture period because by then a very large number of meristems are contributing to the observed increase.

(iii) Although the growth of the main axis from the initial tip meristem is prolonged by the use of ferric citrate, it apparently ceases before that of the lateral roots.

V. The growth of excised tomato roots in media of adjusted initial pH and with ferric citrate as an iron source

The use of ferric citrate instead of ferric sulphate allowed root growth to proceed for a longer period of culture, and since the ferric citrate media showed a similar alkaline pH drift to that characteristic of the standard White's medium, this meant that growth had occurred at higher pH values with the iron supplied as citrate than as sulphate. Experiments No. 9—12 were, therefore, undertaken to study the growth of root tips during 7 days in media containing ferric citrate at 4 concentrations ($\times 1$, $\times 2$, $\times 5$, and $\times 10$ the standard iron concentration) and adjusted by the addition of 'Analar' grade sodium hydroxide to cover a range of pH values above that of the control. Iron was supplied as 'Ferric citrate solution 1 : 1' and the spectrographically standardised ferric chloride solution was used to prepare a control medium containing $\times 2$ the standard iron concentration. Results from these experiments are presented in figure 1 and figure 2. Data for the number of laterals per root are not presented since the values are closely correlated with the length of the main axis. At pH values of 4.9 and below, ferric citrate concentrations above $\times 2$ the standard iron concentration gave media of slightly lower growth-promoting activity than either the control medium or that containing ferric citrate at $\times 1$ and $\times 2$ iron concentration. However, these media containing high ferric citrate concentrations showed a less-marked reduction of their activity at higher pH values. Thus with ferric citrate at $\times 10$ standard iron concentration, the growth at initial pH 5.8—6.0 was at least equal to that occurring in the control medium or in ferric citrate media of initial pH 4.8—5.0 containing $\times 1$ and $\times 2$ iron concentration. Therefore, ferric citrate was able to extend the range of pH over which a high rate of root growth can occur.

The marked decrease in the activity of White's medium which occurs when its initial pH is raised above 4.8 is due to iron precipitation during autoclaving and can be prevented by supplying iron as ferric citrate at

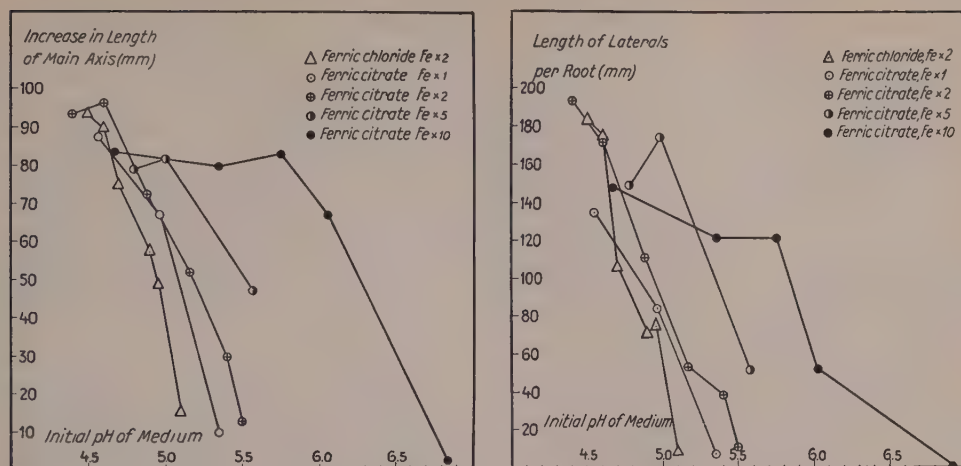
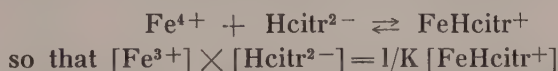


Figure 1 and figure 2. Growth of excised tomato root tips during 7 days' culture in White's medium of different initial pH values and containing ferric chloride or ferric citrate as iron source; iron concentrations expressed as multiples of that in standard White's medium (Exp. No. 9—12).

appropriate concentrations. Visible precipitation of iron does, however, occur in media of high ferric citrate content when the initial pH reaches 5.8—6.0. This may augment the citrate : iron ratio of the solution and thereby, despite the ferric hydroxide precipitate, gives media of higher iron content than those of lower citrate concentration and similar pH. The complex ion FeHcitr^+ will be in equilibrium with Fe^{3+} and Hcitr^{2-} ions thus



The maximum concentration of Fe^{3+} will be controlled by the solubility product of $\text{Fe}(\text{OH})_3$ and will, as already shown, decrease rapidly with increase in pH above 5.0. The concentration of Hcitr^{2-} will depend on total citrate concentration and pH thus:

$$[\text{Hcitr}^{2-}] = \frac{(\text{Total citrate})}{\frac{[\text{H}^+]}{K_2 K_1} + \frac{[\text{H}^+]}{K_2} + 1 + \frac{K_3}{[\text{H}^+]}}$$

Where $K_1 = 8.4 \times 10^{-4}$; $K_2 = 1.8 \times 10^{-5}$ and $K_3 = 4 \times 10^{-6}$. Increasing pH will, therefore, decrease the proportion of the total citrate present as Hcitr^{2-} ; thus at pH 4.5 about 35 per cent of the total citrate will be present as Hcitr^{2-} but this will fall to about 7 per cent at pH 6.5. Increase in pH, by reducing both $[\text{Fe}^{3+}]$ and $[\text{Hcitr}^{2-}]$ will, therefore, reduce the equilibrium concentra-

Table 7. *Growth of excised tomato roots in media containing $\times 5$ standard iron concentration and various proportions of sodium citrate (Exp. No. 13).*

Iron source Molar ratio of iron : citrate shown	Increase in length of main axis (mm)		No. of laterals per root		Dry weight per root (mg)		pH values		
	7 days culture	14 days culture	7 days culture	14 days culture	7 days culture	14 days culture	initial	after 7 days	after 14 days
Ferric chloride	90.6	115.6	25	31.8	2.4	4.75	4.70	5.5	6.46
Ferric Sodium chloride : citrate									
1 : 0.5	97.4	181.3	24.4	43.0	2.35	7.15	4.66	5.93	6.61
1 : 1	89.3	196.8	25.2	48.6	1.95	8.8	4.60	5.80	6.62
1 : 2	60.5	134.4	15.8	40.2	1.00	5.3	4.59	5.70	6.49

tion of the complex ion FeHcitr^+ . If, therefore, high citrate concentration is somewhat toxic but will enable more of the complex to exist at alkaline pH, it would be expected that high citrate: iron ratios would reduce growth-promoting activity at pH values where the iron citrate complex is relatively stable, but would enhance it at higher pH values. In Experiment No. 13 (Table 7) root growth was measured after 7 and 14 days of culture in solutions containing $\times 5$ standard iron concentration and various proportions of sodium citrate. The sodium citrate was prepared by titrating 'Analar' citric acid with 'Analar' sodium hydroxide to pH 4.6. Clearly, within the limits explored in this experiment, the higher the proportion of citrate the higher the ratio of growth in the second 7-day period (pH rising from about 4.6 to about 5.7).

In order to test this idea further culture media covering a range of pH values were prepared with $\times 2$ the standard iron concentration and with iron : citrate molar ratios of 1 : 1 and 1 : 4 and with $\times 5$ iron concentration and ratios 1 : 1 and 1 : 2. These media were inoculated with root tips and the growth measured after 7 days (Experiment No. 14). The results (figures 3 and 4) were as expected in that increase in the citrate : iron ratio decreased growth at pH values approximating to 4.6 but enabled a higher rate of growth to occur at more alkaline pH. The observation that in the region of pH 4.6 the deleterious effect of a 1 : 4 iron : citrate ratio with Fe $\times 2$ is more marked than with a 1 : 2 ratio and Fe $\times 5$ suggests that the determining factor is the iron : citrate ratio rather than the total excess citrate concentration. Hopkins (1930) working with *Chlorella*, observed a similar effect with high citrate: iron ratios and suggested that this was due to lowering of ferric ion concentration by suppression of dissociation of ferric citrate complexes. The

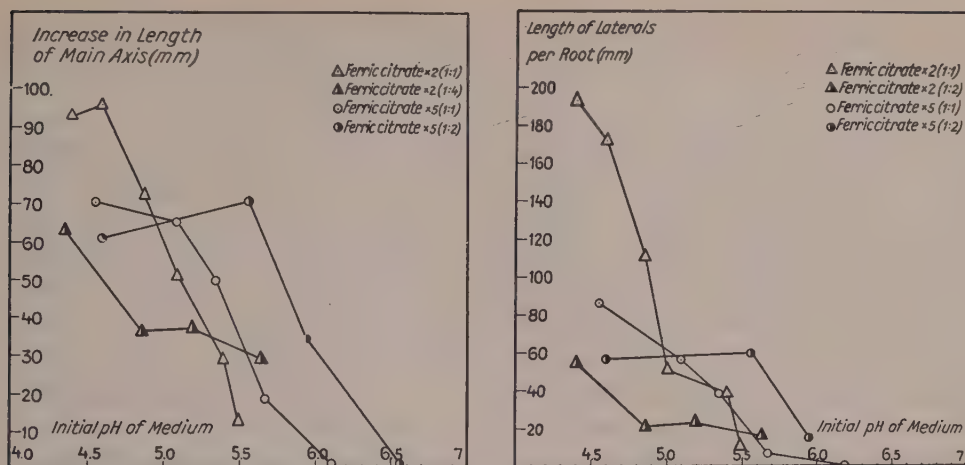


Figure 3 and figure 4. Growth of excised tomato root tips during 7 days' culture in White's medium of different initial pH values and containing ferric chloride at two concentrations in the presence of sodium citrate at the molar ratios indicated (Exp. No. 14).

ability of ferric citrate to promote growth at alkaline pH values strongly supports, however, the view that free ferric or ferrous ions are not the only forms in which iron is utilised, and that in the presence of citrate, the ions of the ferric citrate complex are the immediate iron source. This complex may well act as a donor for the formation of ferric complexes with pectic substances and uronic acids at the surface of the absorbing cells, as has been suggested by Bitcover and Sieling (1951). Jacobson and Overstreet (1947) have suggested that the respiratory activity associated with salt accumulation is required for the formation of ion-binders of the chelate type and Hutner (1948) has postulated that the cell surface is studded with such receptor areas the number of which will, in part, determine the concentration of a particular ion which must be present in the medium to enable the cells to grow and the chemical affinities of which will determine the ability of the cell to absorb metals supplied as complexes. A similar concept underlies Chapman's (1939) postulation of a 'contact exchange' mechanism to explain the ability of plants to use magnetite as a source of iron provided that the root surface is brought into intimate contact with the iron compound. The deleterious effect of excess citrate may, therefore, arise from the formation of other and inactive iron complexes, stable at relatively acid pH, or from stabilisation of the FeHcitr^+ complex to an extent which retards liberation of its iron to the absorbing cells. Alternatively, excess citrate may, by its chelating action, render unavailable other essential elements in the medium (Hutner, 1948).

VI. *Ferrous phosphate and ferric sodium ethylenediamine tetra-acetate as iron sources for excised tomato roots*

Hydrated ferrous phosphate, $\text{Fe}_3(\text{PO}_4)_2 \cdot 8\text{H}_2\text{O}$ at a concentration of 2.5 g/l was used by v. d. Crone (1902) in his original culture solution. Commercial ferrous phosphate contains about 50 per cent w/w of hydrated ferrous phosphate admixed with ferric phosphate and hydrated oxides of iron, and is a blue-grey powder only very slightly soluble in water. The excess solid ferrous phosphate is only slowly oxidised and, therefore, presumably acts as a constant source of ferrous iron to the culture solution. In view of the greater solubility of ferrous as against ferric hydroxide (p. 3), the use of excess ferrous phosphate might be expected to extend, though not markedly, the very limited pH range over which iron is available in White's medium with ferric sulphate as the iron source. Experiment No. 15 was, therefore, undertaken to study the growth-promoting activity at various pH values of White's medium in which the ferric sulphate was replaced by 20 mg/l commercial ferrous phosphate. The medium was adjusted in pH by addition of sodium hydroxide and sterilised by autoclaving. The results are presented in figures 5 and 6. Growth at the favourable pH values was below that usually recorded with White's medium (cf. figures 1 and 2). Using favourable initial pH values and taking measurements at the end of 7 days' incubation, a lower level of growth in the ferrous phosphate as compared with standard White's medium was also observed in Experiment No. 18 (Table 9). Values obtained in Experiment No. 18 for growth after the first 7 days also emphasise that ferrous phosphate is only slightly superior to ferric sulphate as a source of iron in media rendered alkaline as a result of root growth. In the present experiment (No. 15), growth in the presence of ferrous phosphate at pH 5.0 and 5.19, though lower than at more acid pH values, was higher than that obtained with standard White's medium adjusted to these values. There is, therefore, evidence that the use of ferrous phosphate allows of a slight extension towards alkalinity of the pH range over which root growth will occur; a result in agreement with the greater, though low, solubility of ferrous as compared with ferric iron in the critical pH region.

Jacobson (1951) has used ferric potassium ethylenediamine tetra-acetate (Schwarzenbach and Biedermann, 1948) as an iron source for culture solution work, with tomato as one of the test plants. This iron complex proved to be extremely stable even at alkaline pH values and was non-toxic at concentrations giving 25 or less p.p.m. iron. A solution of ferric sodium ethylenediamine tetra-acetate has been prepared in our laboratory by the following slight modification of the method described by Jacobson: — 15 g disodium salt of ethylenediamine tetra-acetic acid was dissolved in 45 ml N NaOH.

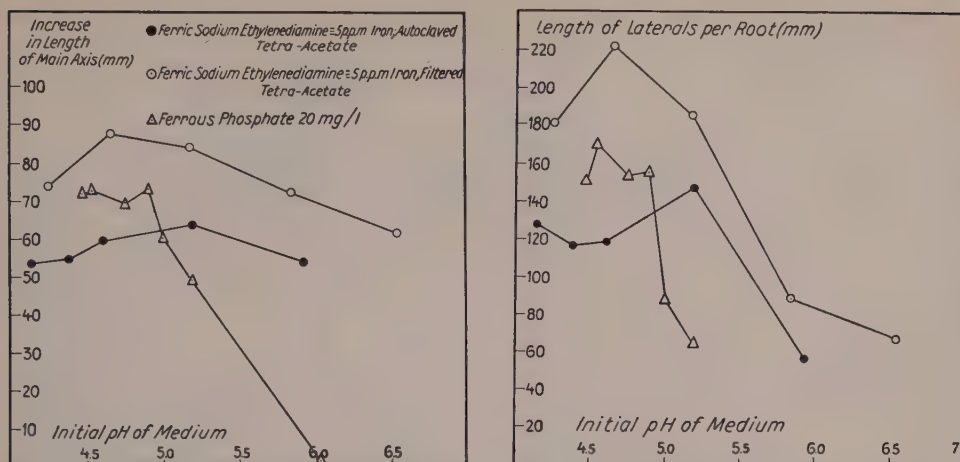


Figure 5 and figure 6. Growth of excised tomato root tips during 7 days' culture in White's medium of different initial pH values and containing as iron source 20 mg/l commercial ferrous phosphate (Exp. No. 15) or ferric sodium ethylenediamine tetra-acetate (=5 p.p.m. Fe) sterilised either by autoclaving or filtration (Exp. No. 16).

To this was added 12.5 g ferrous sulphate $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. The mixture was then diluted, adjusted to pH 4.6 by the addition of alkali, made up to 500 ml with distilled water and aerated overnight using a sintered-glass aerator.

A study has been made of the growth-promoting activity of White's medium when adjusted to various pH values and modified by replacing the ferric sulphate by ferric sodium ethylenediamine tetra-acetate at a concentration equivalent to 5 p.p.m. iron (approximately $\times 7$ standard iron concentration) (Experiment No. 16). Media containing this iron complex and adjusted in pH by the addition of sodium hydroxide, showed a more pronounced acid drift on autoclaving than had occurred with the other iron sources tested. When pH values were initially 5.5 or higher, this drift was of the order of one pH unit. It was thought that this might indicate a decomposition of the complex during heat-sterilisation and two series of media were, therefore, prepared: (a) 'Complex-autoclaved' series: — complete medium, appropriately adjusted in pH, sterilised by autoclaving and pH values determined on replicate flasks at time of inoculation. (b) 'Complex-filtered' series: — medium at 1.25 normal strength with iron omitted, appropriately adjusted in pH, distributed 40 ml per flask and sterilised by autoclaving; a solution of the iron complex at $\times 5$ the desired final concentration sterilised by filtration and then added aseptically, 10 ml per flask, to the autoclaved iron-free medium and pH values determined on replicate flasks of completed medium at time of inoculation.

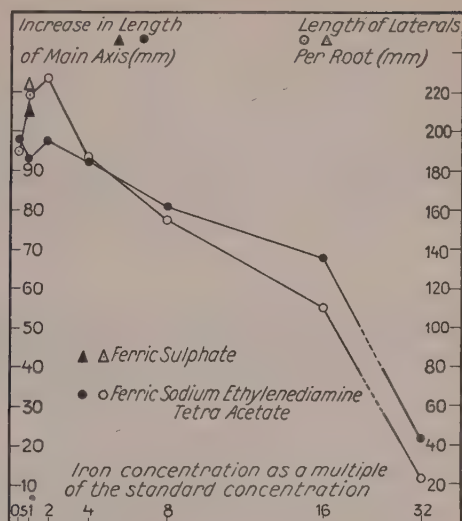
Table 8. *Method of preparing media used in Experiment No. 17. The volumes of solutions per culture flask are indicated.*

Iron source	Concentration of iron relative to the concentration in standard White's medium	Volume (ml) of $\times 5$ normal concentration 'iron-omitted' medium	Volume (ml) double-distilled water	Volume (ml) of filtration-sterilised solution of Ferric sodium ethylenediamine tetra-acetate		pH of medium	
				Soln. A	Soln. B	initial	final
Ferric sodium ethylenediamine tetra-acetate	$\times 32$	10	nil	40		4.84	4.30
	$\times 16$	10	20	20		4.86	4.61
	$\times 8$	10	30	10		4.86	4.95
	$\times 4$	10	35	5		4.75	5.50
	$\times 2$	10	20		20	4.50	5.77
	$\times 1$	10	30		10	4.60	5.86
	$\times 0.5$	10	35		5	4.58	5.83
Ferric sulphate	$\times 1$					4.60	5.56

The results of Experiment No. 16 are shown in figures 5 and 6. The addition of ferric sodium ethylenediamine tetra-acetate to White's medium extends the pH range for root growth to the alkaline side with very little reduction of growth-promoting activity. Comparison of the level of growth in the 'Complex-autoclaved' and 'Complex-filtered' series strongly supports the view that some decomposition of the complex occurs on autoclaving in White's medium. In the 'Complex-filtered' series, the level of growth was similar to that normally obtained in White's medium and was maintained over a wider pH range.

In view of the possible toxicity of high concentrations of this complex, Experiment No. 17 was designed to study the growth-promoting activity of media of standardised initial pH containing the complex over a range of concentrations. 'Iron-omitted' medium was sterilised by autoclaving and solutions of the iron complex were sterilised by filtration and added aseptically to the autoclaved medium. Solution A of ferric sodium ethylenediamine tetra-acetate was $\times 8$ the concentration of Solution B. The preparation of the experimental media is detailed in Table 8. The concentrations of iron shown in this Table can be readily converted to p.p.m. iron on the basis that $\times 1$ standard concentration equals approximately 0.7 p.p.m. iron. Table 8 also shows the pH values of the media prior to inoculation and at the time of harvesting the roots. The results of this experiment, together with those for standard medium prepared and inoculated on the same occasion, are shown in fig. 7. They show that at $\times 1$ and $\times 2$ standard iron concentration, ferric sodium ethylenediamine tetra-acetate supported growth equal to that with ferric sulphate as the iron source. At $\times 4$ and $\times 8$ standard iron concentration,

Figure 7. Growth of excised tomato root tips during 7 days' culture in White's medium containing as iron source filtration-sterilised ferric sodium ethylenediamine tetra-acetate at various concentrations (see Table 8, Exp. No. 17).



the complex was slightly toxic, and at still higher concentrations, the toxicity became marked. However, when used at appropriate concentration, this iron complex enables medium to be prepared which is not only equal in growth-promoting activity to White's medium of favourable pH, but is capable of supporting a high rate of root growth over a considerably wider pH range. It represents a satisfactory alternative to ferric citrate in maintaining iron availability at high pH values. The pH stability of the iron complex with ethylenediamine tetra-acetate implies that it must remove ferric ions from solution as effectively, or even more effectively, than does citrate. Its suitability as an iron source, therefore, supports the view that complexes active in this way are able to serve as direct donors of iron for root-growth.

In order to compare media containing ferrous phosphate and ferric sodium ethylenediamine tetra-acetate with standard medium and with medium containing ferric citrate ('Ferric citrate solution 1:1'), Experiment No. 18, similar in plan to Experiment No. 8, was undertaken. The growth achieved in these four media was determined at intervals during 28 days, 15 cultures from each medium being harvested on each occasion. The results are given in Table 9. Both ferric citrate and ferric sodium ethylenediamine tetra-acetate gave similar high values for dry weight increase during the extended culture period. The complexes were used at concentrations above the optimum for high initial growth rate but their ability to maintain the iron supply at more alkaline pH values resulted in a high rate of dry weight increase after the 7th day. As in Experiment No. 8 (Table 6), with media capable of maintaining the iron availability, the main axis ceased to grow whilst dry

Table 9. *Growth of excised tomato roots in various media* (Exp. No. 18).

Feature measured	Iron source and concentration	Period of Culture (days)				
		0	7	14	21	28
Increase in length of main axis (mm)	$\text{Fe}_2(\text{SO}_4)_3 \cdot \text{Fe} \times 1$		89	138	136	154
	Ferrous phosphate 20 mg/l		74	139	133	139
	Ferric sodium ethylenediamine tetraacetate, $\text{Fe} \times 5$		69	130	144	149
	Ferric citrate, $\text{Fe} \times 5$		75	159	168	162
No. of primary laterals per root	$\text{Fe}_2(\text{SO}_4)_3 \cdot \text{Fe} \times 1$		33	53	55	66
	Ferrous phosphate 20 mg/l		21	48	55	61
	Ferric sodium ethylenediamine tetraacetate, $\text{Fe} \times 5$		20	37	38	39
	Ferric citrate, $\text{Fe} \times 5$		20	42	45	42
Dry weight per root (mg)	$\text{Fe}_2(\text{SO}_4)_3 \cdot \text{Fe} \times 1$		1.9	5.0	5.7	7.3
	Ferrous phosphate 20 mg/l		1.5	5.0	6.5	6.5
	Ferric sodium ethylenediamine tetraacetate, $\text{Fe} \times 5$		1.7	8.6	14.9	22.1
	Ferric citrate, $\text{Fe} \times 5$		1.5	8.2	14.6	20.3
pH of culture media	$\text{Fe}_2(\text{SO}_4)_3 \cdot \text{Fe} \times 1$	4.1	5.3	6.3	6.8	6.7
	Ferrous phosphate 20 mg/l	4.6	5.3	6.6	6.8	6.7
	Ferric sodium ethylenediamine tetraacetate, $\text{Fe} \times 5$	4.4	5.1	6.5	7.2	7.2
	Ferric citrate, $\text{Fe} \times 5$	4.5	5.5	6.5	7.2	7.2

weight was still increasing rapidly. In medium containing ferrous phosphate, root growth was initially inferior and later in the culture period slightly superior to that attained in standard medium. This result confirms the conclusions reached in Experiment No. 15.

VII. *The influence of root growth on iron availability*

Even when due allowance was made for the test-to-test variability encountered in root culture experiments (Boll and Street, 1951), a comparison of the results obtained using extended culture periods (Tables 4—7 and 9) with those using a 7-day culture period and media the pH of which had been raised by additions of alkali (figures 1—4) appeared to strengthen a previously advanced view (Street, McGonagle and Lowe, 1951). This is, that medium whose pH has been raised by root growth has a higher growth-promoting activity than new medium adjusted to the same pH by addition of alkali. Experiments 19 and 20 were undertaken in an attempt to examine this view more critically. Medium was inoculated with root tips, incubated 7 days, the roots harvested aseptically, 5 sample flasks of medium removed for pH determinations and the remaining flasks reinoculated with tips. This pro-

Table 10. *Excised tomato roots grown from root tip inocula in successive 7-day culture periods in the same sample of standard White's medium (Exp. No. 19).*

Medium	Initial pH	Increase in length of main axis (mm)	No. of laterals per root	Length of laterals per root (mm)
1. New Medium.....	4.23	96	46	230
2. Medium from which roots described under 1. above had been harvested	5.50	6.9	1.7	2.0

cedure was, in Experiment No. 20 repeated after a further 7 days' incubation so that in this case each 50 ml medium was used to support three successive tip passages. In both experiments 50 cultures were inoculated, this number being reduced at each harvest by removal of the flasks for pH determinations so that in Experiment No. 20 the third passage comprised 40 cultures.

The results of Experiment No. 19, in which standard White's medium containing ferric sulphate was used, are shown in Table 10. Medium which had drifted to pH 5.5 as a result of root growth had almost no growth-promoting activity when assayed by inoculation with root tips. Any mitigation during 'staling' of the decrease in growth-promoting activity which results from rise in pH is no longer evident in medium of pH 5.5.

The results of Experiment No. 20, in which ferric citrate at $\times 2$ standard iron concentration was used as iron source, are compared with those of Experiment No. 10 in Table 11. It is clear from these results that the 'staled' medium was of higher growth-promoting activity than new medium adjusted to the same pH by addition of alkali. The effect was very striking in media of pH 5.5 and evident even at pH 6.1–6.2.

The following possible explanations for the observed differences in growth-promoting activity between 'staled' and 'alkali adjusted' media were then considered: — that (a) the increase in sodium concentration involved in pH adjustment has a deleterious effect; (b) autoclaving at the higher pH values either leads to the establishment of the equilibrium iron concentration which is only reached very slowly at 27°C incubation, or causes other unfavourable chemical changes; (c) root growth results in a change in the composition of the medium which mitigates the unfavourable effect of increasing pH.

From a consideration of the high growth achieved in the citrate-containing media described in this paper, and from other unpublished data, it can be stated that the changes in sodium concentration involved in the pH adjustments are without significant effect upon root growth.

The view that autoclaving at the higher pH values leads to unfavourable chemical changes in the medium is not borne out by the results of Experiments 9–12 and 14, where media with high citrate content are shown to be

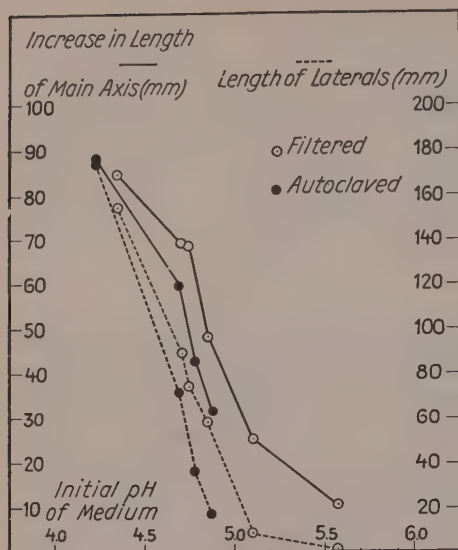


Figure 8. *The effect of sterilisation by autoclaving as compared with sterilisation by filtration in the growth-promoting activity of standard White's medium adjusted by alkali to have, after sterilisation, various pH values. Activity assayed by the growth of excised tomato root tips during 7 days' culture (Exp. No. 21).*

able to support good growth although autoclaved at high pH. Therefore, it seemed much more likely that growth occurs in standard medium and in media of low ferric citrate concentration at higher pH values if these arise as a result of root growth, because hydrolysis and precipitation of iron take place slowly at the incubation temperature, or because substances arise during growth which mitigate the unfavourable effect of pH by forming soluble complexes with iron, or for both reasons.

When ferric sulphate is dissolved in water as recommended by White (1943), it gives an opalescent solution which is unstable and yields a brown precipitate on standing. A clear yellow solution of greater stability is obtained by dissolving the ferric sulphate in the solution of sucrose. Even when so prepared, White's medium causes a brown colouration of a No. 4 sintered-glass filter. The colouration is removed by addition of hydrochloric acid, and the resulting filtrate gives a strong reaction for ferric iron with potassium thiocyanate (Sandell, 1944). Determination of iron in filtered White's medium using the thiocyanate method and measuring absorption at 480 m μ in a spectrophotometer, shows it to contain only 60–75 per cent of the theoretical iron content. When autoclaved medium of pH within the range 4.6–4.75 was filtered through a No. 32 Whatman paper, it contained only 25–40 per cent of the theoretical amount of iron. Medium of pH 5.4 when autoclaved and filtered contained no detectable iron and, in fact, had a higher transmission than non-autoclaved 'iron-omitted' medium. At all the pH values tested, the amount of iron in solution was lower in autoclaved medium than

Table 11. *Excised tomato roots grown from root tip inocula for 7-day culture periods in medium rendered alkaline by previous root growth (Exp. No. 20) and in corresponding new medium (ferric citrate at $\times 2$ standard iron concentration) adjusted by alkali to similar pH and sterilised by autoclaving (Exp. No. 10).*

Culture Medium	Initial pH	Increase in length of main axis (mm)	No. of laterals per root	Length of laterals per root (mm)
1. New Medium	4.58	85.5	22.9	137
New Medium	5.5	12.4	3.9	10.9
2. Medium from which roots shown in 1. above had been harvested	5.5	53.4	13.5	53.5
New Medium	6.18	nil	nil	nil
3. Medium from which roots shown in 2. above had been harvested	6.11	16.5	3.75	13.2

in non-autoclaved medium stored at room temperature for the same period. Experiments were, therefore, undertaken to compare, at various pH values, the effect of autoclaving with that of filter-sterilisation upon the growth-promoting activity of White's medium (Experiment No. 21) and medium containing ferric citrate at $\times 2$ standard iron concentration (Experiment No. 22). The results of Experiment No. 21, shown in figure 8, indicate that with White's medium of pH 4.7 or above, sterilisation by filtration gives medium of higher growth-promoting activity than sterilisation by autoclaving. However, even media sterilised by filtration have only very low activity if their pH is 5.1 or over. Media containing 'Ferric citrate solution 1:1' at $\times 2$ standard iron concentration (Experiment No. 22) also showed the beneficial effect of filter-sterilisation at the higher pH values employed (Table 12). The loss in growth-promoting activity by autoclaving at pH values over 5.0 was quantitatively sufficient to account for the apparent beneficial effect of 'staling' noted in Experiment No. 20 (Table 11). This loss of growth-

Table 12. *Effect of the method of sterilisation of the medium on the growth of excised tomato root tips in media containing 'Ferric citrate solution 1:1' at $\times 2$ standard iron concentration and adjusted in pH by addition of alkali (Exp. No. 22).*

Method of sterilisation	Initial pH	Increase in length of main axis (mm)	No. of laterals per root	Length of laterals per root (mm)
Autoclaving	4.96	47.6	9.8	25.1
Autoclaving	5.19	37.1	7.4	24.6
Filtration	5.37	61.7	10.6	39.3
Autoclaving	6.21	nil	nil	nil
Filtration	6.24	15.5	2.8	3.7

Table 13. *Effects on the growth of excised tomato roots of the concentration of ferric sulphate in White's medium and of the addition of monosaccharides (0.075 % dextrose and 0.15 % laevulose) to the media.*

Experi- ment	mg/l Ferric sulphate	Monosaccharides pre- sent (+) or absent (—) Dextrose only, + D. Laevulose only, + L.	Increase in length of main axis (mm)	No. of laterals per root	Total length of laterals per root (mm)
23	5.0	—	83.0	31.4	219.6
	2.5	—	92.7	35.4	199.2
	1.0	—	44.9	18.6	72.3
	0.5	—	39.9	13.7	36.7
	0.2	—	2.8	2.2	5.8
	nil	—	2.2	nil	nil
	nil	+	12.6	4.9	5.8
24	2.5	—	83.0	32.1	179
	2.5	+	76.0	29.1	147
	1.0	—	66.2	22.2	75
	1.0	+	52.5	19.0	91
	0.4	—	11.0	2.1	2.6
	0.4	+	55.2	21.3	75
	0.2	—	10.0	3.1	3.3
	0.2	+	46.0	16.1	41.5
	nil	—	6.1	1.5	1.2
	nil	+	27.3	9.0	17
25	0.4	—	3.1	0.7	
	0.4	+ D	8.7	2.1	
	0.4	+ L	18.2	5.1	
	0.2	—	1.2	nil	
	0.2	+ D	3.4	0.6	
	0.2	+ L	12.3	2.9	

promoting activity by autoclaving would seem to be due to acceleration of iron precipitation during the sterilisation process.

Earlier work (Street, McGonagle and Lowe, 1951) had led to the conclusion that although excised tomato root tips are adversely affected by inclusion of monosaccharides in the medium, it appears that their natural accumulation during growth does not act as a 'staling' factor, but rather seems to lessen the injurious effects of alkaline pH. This conclusion was based upon the results of attempts to prepare an artificial 'staled' medium by adding dextrose and laevulose to standard medium rendered alkaline by addition of sodium hydroxide (Street, McGonagle and Lowe, 1951. Table 4). The monosaccharides were added in quantities approximating to the amount arising from 7-days culture (Dormer and Street, 1949). The possibility, therefore, presented itself that the naturally-formed monosaccharides might be responsible for retaining iron in solution in medium rendered alkaline by root growth. However, it was first necessary to demonstrate that any beneficial effect of added monosaccharides was not due solely to iron in the form

Table 14. *Comparison of the growth-promoting activity of monosaccharides (0.075 % dextrose+0.15 % laevulose) assayed in 'iron-omitted' White's medium and the effect of purification with 'Bio-deminrolit F' on this activity (Exp. No. 26).*

Medium	Increase in length of main axis (mm)	No. of laterals per root	Length of laterals per root (mm)
'Iron-omitted' medium	1.7	3	1.1
» » » + monosaccharides ...	69.5	29.6	77.2
» » » + purified monosaccharides	6.6	2	2.8

of a complex added as a contaminant in these sugars. Burk, Lineweaver and Horner (1932) noted that iron contamination of the dextrose affected the growth-promoting activity of a synthetic medium used for *Azotobacter* culture and that this iron was not so completely precipitated as the same concentration of inorganic iron at pH values in the range 6.0—7.2. The dextrose, and particularly the laevulose sample, used in our work contained readily-detectable traces of iron when tested with potassium thiocyanate, whereas any iron contamination in the sucrose was at the limit of sensitivity of the test. Working at favourable pH values, it was possible to demonstrate a stimulatory effect of low concentrations of added monosaccharides upon root growth in White's medium containing ferric sulphate at concentrations of 0.4 mg/l or lower (results of Experiments No. 23, 24, and 25 presented in Table 13). These experiments not only demonstrated the absence of growth in the absence of added iron, and the critical nature of the iron concentration capable of supporting optimum growth, but also showed that our monosaccharide samples, used at a concentration slightly toxic in standard medium, were stimulatory in medium of low iron content. Therefore, to study more critically the effect of monosaccharides, it was necessary to reduce the iron contamination in our samples. This was effected by the use of the ion exchange resin 'Bio-Deminrolit F' (Permutit Co. Ltd., London, W 4.). The effect of the addition of 0.75 per cent dextrose+1.5 per cent laevulose to 'iron-omitted' standard medium was tested using (i) commercial samples; (ii) the same samples after passing through a 9" column of 'Bio-Deminrolit F' as a mixed solution containing 3.75 per cent dextrose and 7.5 per cent laevulose (Experiment No. 26, Table 14). The monosaccharide samples used had a particularly striking growth-promoting effect which was almost completely lost by treatment with the ion exchange resin. The purified solution, when added to standard medium containing iron, had only the same mild toxicity as the commercial samples.

Experiments No. 27—30 were then undertaken to test the effect of the purified monosaccharides on the growth-promoting activity of White's

Table 15. *Effects on the growth of excised tomato roots of additions of monosaccharides (0.075 % dextrose and 0.15 % laevulose) to White's medium adjusted in pH and sterilised by filtration (Exp. No. 27—30).*

Exp. No.	Medium	Increase in length of main axis (mm)	No. of laterals per root	Length of laterals per root (mm)	pH Values	
					Initial	Final
27	Standard	58	26	72	4.9	4.8
	Standard + monosaccharides	63	26	65	4.9	5.1
	Standard + purified monosaccharides	67	31	91	4.9	5.3
28	Standard	39	12.5	32	5.0	4.9
	Standard + monosaccharides	51	23	47	4.9	5.2
	Standard + purified monosaccharides	44	23	63	5.0	5.0
29	Standard	28	15	60	5.1	4.8
	Standard + purified monosaccharides	58	22	64	5.1	5.3
30	Standard	3.3	nil	nil	5.8	5.5
	Standard + monosaccharides	3.5	nil	nil	5.8	5.4
	Standard + purified monosaccharides	3.1	nil	nil	5.7	5.4

medium whose pH had been raised by addition of alkali. White's medium, when containing added monosaccharides, shows on autoclaving a variable acid drift suggesting the formation of sugar acids from the monosaccharides. Such acids would form complexes with ferric iron and it was thought that this might have been the explanation of the earlier observed beneficial effect of added monosaccharides when using media having, after autoclaving, pH values of 5.0—5.2 (Street, McGonagle and Lowe, 1951). In order to eliminate effects of heat-sterilisation and to obtain standard and test media of the same initial pH, sterilisation by filtration was used in the present experiments. The results, presented in Table 15, confirmed that the addition of monosaccharides to White's medium of pH 4.9—5.1 has a beneficial effect and showed that this effect was independent of the iron content of the sugars. At a pH of 5.7—5.8 the added monosaccharides had no beneficial effect. The inability of 'staled' White's medium of pH. 5.5 to support the growth of new tip inocula (Experiment No. 19, Table 10) is probably, therefore, explained on the basis that at this pH the accumulated monosaccharides are unable to maintain iron availability.

It still remained necessary to explain the increases in dry weight of roots when allowed to remain in White's medium whose pH had risen as a result of root growth to values in excess of 5.5 (Experiments No. 8 and 18, Tables 6 and 9). If the conclusions reached above are valid such media should be

Table 16. *The further growth of 7-day old excised tomato roots cultured for 7 days in 'staled' White's medium and in new standard White's medium with iron omitted (Exp. Nos. 30 and 31).*

Exp. No.	Medium	pH values of medium		Increase in length of main axis (mm)	Increase in number of laterals per root	Increase in length of laterals per root (mm)	Increase in dry weight per root (mg)
		Initial	Final				
31	'Staled' medium	4.98	6.41	26.1	20.3	135	2.48
	Iron-omitted new medium	4.33	5.73	9.1	12.3	62	1.93
32	'Staled' medium	5.37	6.51	23.4	22.3	53	2.17
	Iron-omitted new medium	4.49	6.45	32.0	21.7	88	2.72

devoid of available iron and the observed growth must have been possible, due to utilisation of iron accumulated from the medium during the beginning of the culture period when the pH was compatible with iron availability. Experiments No. 31 and 32 have been undertaken to test this. In both cases 75 tip cultures were established in standard White's medium. After 7 days incubation 25 cultures were harvested, the roots measured and the pH of the medium determined. 25 cultures were incubated for a further 7 days. Roots from the third set of 25 cultures were transferred aseptically to new 'iron-omitted' medium and incubated for 7 days. The increases in linear measurements and dry weight of the roots and the pH of the medium during the second 7 day culture period were then determined for both the 'staled' and the 'iron-omitted' medium. In Experiment No. 31 the roots were grown at $25.8^{\circ} \pm 0.5^{\circ}$ during the first 7 days in order to yield medium whose pH would not have risen beyond the range at which monosaccharides have a beneficial effect on iron availability. All other cultures in these experiments were grown at the standard incubation temperature of $27^{\circ} \pm 0.5^{\circ}$. The results, presented in Table 16, show that whilst the 'staled' medium of pH 4.98 is superior to 'iron-omitted' medium this is not the case with 'staled' medium of pH 5.4. Roots which have grown for 7 days in White's medium are, therefore, for a time capable of a further slow rate of growth in a medium devoid of iron. This is presumably due either to iron reserves being translocated to the meristems or to complexed iron being liberated into the medium from the older cells and then re-absorbed at the growing points. White (1937) has recorded that roots stored in medium in which they had ceased to grow may suddenly develop new laterals which in turn become inhibited and that this process may be repeated. This behaviour may be due to a discontinuous liberation of complexed iron from groups of cells as they become senescent.

Discussion

The results presented in this paper show that the pH drift towards alkalinity occurring in White's medium as a result of root growth acts as a 'staling' factor primarily by causing iron precipitation and thereby rendering the medium iron deficient. When this effect is overcome by supplying iron as a stable complex, the medium supports growth for a much longer time despite the drift in pH to neutrality or even mild alkalinity. Experiments with media containing a stable iron complex at appropriate concentration and adjusted in pH, show that excised tomato roots can be grown satisfactorily from root tip inocula over a much wider range of initial pH than has hitherto been possible. Study of root growth in such media does, however, strongly support the conclusion that the growth of the main axis is limited and ceases whilst the laterals are still growing at an appreciable rate. It is suggested that this may arise from one or a combination of the following causes. (i) The inhibition of growth resulting from changes occurring in the medium takes time to manifest itself. For instance, this could occur if some 'staling' product is slowly accumulated by the meristem and has to reach a critical concentration before it markedly reduces and ultimately prevents meristematic activity. (ii) The new medium contains some toxic factor which operates in a similar manner to that postulated above. (iii) The meristem of the main axis, and later the meristems of the laterals, are inhibited by an internal factor which no alteration in medium composition can overcome and that this is produced either in the meristem itself, or in the recently initiated lateral meristems, or in the mature root tissues. (iv) A change occurs in each meristem as it 'ages' which renders it susceptible to inhibition by 'staling' factors. This problem will be further examined in conjunction with studies now proceeding on the possibility of repeatedly subculturing individual meristems.

The use of iron complexes, by increasing the amount of root growth in a given volume of medium, makes it possible to permit 'staling' to go on for a longer period and thereby will enable further examination of the changes in medium composition which occur as a result of root growth. White's medium modified by the inclusion of these iron complexes will also be of particular value in studying vitamin and micro-element requirements by increasing the possibility of depleting any of the materials under test which may be carried over in the inoculum or be present as a contaminant in the medium.

Using White's medium it was concluded (Street and Lowe, 1950) that excised tomato roots are only able to utilise sucrose over a very narrow pH range. This is no longer true. Further, by using either ferric citrate or ferric

sodium ethylenediamine tetra-acetate, it will now be possible to study the effect of nutrient treatments on excised tomato roots over an extended range of pH values. This increase in the scope of the physiological investigations which can be undertaken by the technique of excised root culture will undoubtedly lead to important modifications of our present views, based as they are on the responses of excised roots in medium which becomes iron deficient unless the pH conditions are rigidly controlled.

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Summary

1. The growth-promoting activity of White's medium 'staled' by the growth of excised tomato roots and then adjusted to favourable pH, is not increased by the addition of sucrose or vitamins or the 'solution of inorganic salts'. Addition of ferric sulphate, however, causes a marked increase in growth-promoting activity. With medium 'staled' by supporting root growth for only 7 days this addition almost restores the activity to that of new medium.
2. The alkaline drift occurring in White's medium as a result of root growth causes iron to be precipitated and the consequent iron deficiency cannot be prevented by increasing the initial concentration of ferric sulphate. pH drift operates as a 'staling' factor primarily by its effect on iron availability.
3. White's medium modified by supplying the nitrogen as ammonium nitrate does not support as high an initial growth rate as the standard medium, but since its pH does not increase during culture, no iron deficiency intervenes to cause an early decline in its growth activity.
4. Other soluble inorganic ferrous and ferric salts can be effectively substituted for the ferric sulphate of White's medium but they do not prevent the development of iron deficiency as the pH increases. A slight extension of the pH range over which iron is available can be achieved by using ferrous phosphate which acts as a constant source of ferrous ions. Iron supplied as ferric citrate or as ferric sodium ethylenediamine tetra-acetate remains available over a much wider pH range and their use as iron sources allows root growth to continue for much longer periods than in standard White's medium.
5. Commercial ferric citrate scales were found to contain copper at a concentration which, because of its toxicity, seriously limited the con-

centration of iron which could be supplied in this form. Using a specially prepared ferric citrate solution it was found that either increasing the ferric citrate concentration up to $\times 10$ the standard iron concentration or increasing the citrate:iron ratio reduced the growth-promoting activity of medium of low pH but enhanced the level of growth at the higher pH values tested.

6. Ferric sodium ethylenediamine tetra-acetate undergoes some decomposition when autoclaved in White's medium, but if the medium is sterilised by filtration, the complex is non-toxic up to $\times 4$ the standard iron concentration and supports growth up to at least pH 6.5.
7. From a study of the growth achieved when iron is supplied in the form of these complexes at different concentrations and at different pH values, it is suggested that the complex ions act as direct donors of ferric iron to chelating substances at the surface of the absorbing cells.
8. It was found that sterilisation of media by autoclaving, as compared with filter-sterilisation, caused, particularly at pH values higher than 4.8, a loss of iron by precipitation from both standard medium and medium containing ferric citrate. The effect was most marked with standard medium.
9. The development of iron deficiency in White's medium as a result of root growth is, in the pH range 4.9—5.1, checked by the simultaneous formation of monosaccharides. Monosaccharides added to new medium of initial pH within this range, in quantities such as arise during the 7-day culture period, increase its growth-promoting activity. By purification of the commercial samples of dextrose and laevulose used it has been shown that this effect is not due to iron contamination in the sugar samples.
10. Roots developed in standard White's medium are able to continue a slow rate of growth for some time after the rise in pH of the medium has rendered iron unavailable. It is suggested that this is made possible by utilisation of iron absorbed earlier in the culture period.
11. The bearing of the results on the technique and potentialities of excised root cultures for physiological research is discussed.

References

1. Bitcover, E. H. & Sieling, D. H.: Effect of various factors on the utilisation of nitrogen and iron by *Spirodela polyrhiza* (L.) Schleid. — *Plant Physiol.* 26: 290. 1951.
2. Boll, W. G. & Street, H. E.: Studies on the growth of excised roots. I. The stimulatory effect of molybdenum and copper on the growth of excised tomato roots. — *New Phytol.* 50: 52. 1951.
3. Bonner, J.: On the growth factor requirements of isolated roots. — *Am. J. Bot.* 27: 692. 1940.

4. — & Addicott, F.: Cultivation *in vitro* of excised pea roots. — Bot. Gaz. 99: 144. 1937.
5. — & Devirian, P. S.: Growth factor requirements of four species of isolated roots. — Am. J. Bot. 26: 661. 1939.
6. Burk, D., Lineweaver, H. & Horner, C. K.: Iron in relation to the stimulation of growth by humic acid. — Soil Sci. 33: 413. 1932.
7. Chapman, H. D.: Absorption of iron from finely ground magnetite by citrus seedlings. — Soil Sci. 48: 309. 1939.
8. Clark, N. A.: Green plants and the reactions of iron. — Proc. Iowa Acad. Sci. 43: 185. 1936.
9. van den Crone, G.: Ergebnisse von Untersuchungen über die Wirkung der Phosphorsäure auf die höhere Pflanze. — Sitz. d. Niederrhein. Gesellsch. z. Bonn. 167. 1902.
10. Dormer, K. J. & Street, H. E.: The carbohydrate nutrition of tomato roots. — Ann. Bot. 13: 199. 1949.
11. Halvorson, H. O. & Starkey, R. L.: Studies on the transformations of iron in nature. I. Theoretical considerations. — J. Phys. Chem. 31: 626. 1927.
12. Hoagland, D. R.: Relation of the concentration and reaction of the nutrient medium to the growth and absorption of the plant. — J. Agric. Res. 18: 73. 1919.
13. Hopkins, E. F.: Iron-ion concentration in relation to growth and other biological processes. — Bot. Gaz. 89: 209. 1930.
14. — & Wann, F. B.: Relation of hydrogen-ion concentration to growth of *Chlorella* and the availability of iron. — Bot. Gaz. 81: 353. 1926.
15. Hutner, S. H.: Essentiality of constituents of sea water for growth of a marine diatom. — Trans. New York Acad. Sci. Ser. II. 10: 136. 1948.
16. Jacobson, L.: Maintenance of iron supply in nutrient solutions by a single addition of ferric potassium ethylenediamine tetra-acetate. — Plant. Physiol. 26: 411. 1951.
17. Jacobson, L. & Overstreet, R.: A study of the mechanism of ion absorption by plant roots using radioactive elements. — Am. J. Bot. 34: 415. 1947.
18. Jones, L. H. & Shive, J. W.: Effect of ammonium sulphate upon plants in nutrient solutions supplied with ferric phosphate and ferrous sulphate as sources of iron. — J. Agric. Res. 21: 701. 1921.
19. — — Influence of ammonium sulphate on plant growth in nutrient solutions and its effect on hydrogen-ion concentration and iron availability. — Ann. Bot. 37: 355. 1923.
20. Langford, O. E., & Quinan, J. R.: A spectrophotometric study of the reaction of ferric iron and citric acid. — J. Am. Chem. Soc. 70: 2900. 1948.
21. Sandell, E. B.: Colorimetric determination of traces of metals. — Interscience. New York. 1944.
22. Schwarzenbach, G. & Biedermann, M.: Komplexe IX. Titration von Metallen mit Äthylenediamintetraessigsäure H_4Y . Endpunktsindikation durch pH-Effekte. — Helv. Chim. Acta. 31: 459. 1948.
23. Street, H. E. & Lowe, J. S.: The carbohydrate nutrition of tomato roots. II. The mechanism of sucrose absorption by excised roots. — Ann. Bot. 14: 307. 1950.
24. — McGonagle, M. P. & Lowe, J. S.: Observations on the 'staling' of White's medium by excised tomato roots. — Physiol. Plant. 4: 592. 1951.
25. — & McGregor, S. M.: The carbohydrate nutrition of tomato roots. III. The effect of external sucrose concentration on the growth and anatomy of excised roots. — Ann. Bot. 16: 185. 1952.

26. Thomas, W.: Availability of mineral plant nutrients in relation to the degree of dispersion. — *Soil Sci.* 27: 249. 1929.
27. Tottingham, W. E. & Rankin, E. J.: The availability of iron in nutrient solutions for wheat. — *Am. J. Bot.* 10: 203. 1923.
28. White, P. R.: Survival of isolated tomato roots at sub-optimal and supra-optimal temperatures. — *Plant Physiol.* 12: 771. 1937.

Nature and Formation of Phenol Oxidases in *Polyporus zonatus* and *P. versicolor*

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Polyporus zonatus Fr. and *P. versicolor* Fr. belong to the typical white rot fungi. When cultivated on agar containing phenolic substances (gallic or tannic acid etc.), the mycelia secrete phenol oxidases into the medium, thereby causing marked coloured zones. During earlier work on these species and other lignin-decomposing hymenomycetes, the authors found phenol oxidases to be secreted even into synthetic nutrient solutions which did not contain any phenols. However, the composition of the medium in certain cases greatly affected the amount of enzyme secreted. The present paper deals with the properties of the phenol oxidases of *P. zonatus* and *P. versicolor* and with some factors influencing the formation of these enzymes.

Materials and methods

A pure culture of *Polyporus zonatus* was kindly supplied by Prof. E. Björkman, Stockholm. A strain of *P. versicolor* was isolated from spores.

In the experiments with *P. zonatus*, the following basic medium was used: Glucose 10 g, asparagine 1.5 g, KH_2PO_4 5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, NaCl 0.25 g, Fe, Zn, and Cu 1 p.p.m., Mn 3 p.p.m., Ca 20 p.p.m., aneurin 50 μg , distilled water (and different additions) to 1000 ml. pH after autoclaving 4.6.

P. versicolor (Table 1—2) was cultivated on the following basic medium: Glucose 20 g, NH_4Cl 2 g, KH_2PO_4 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, trace elements and aneurin as above, distilled water to 1000 ml. In the pH experiment (Table 4) 5 g KH_2PO_4 were added and the NH_4Cl was replaced by 2.45 g asparagine.

In the pH experiments, the pH of the solutions was regulated by adding 0.1 *N* HCl or 0.1 *N* NaOH.

Each flask contained 25 ml solution.

Enzyme extracts of the mycelia and solutions from *P. zonatus* were prepared as described by Lindeberg and Holm (1952). In the experiments with *P. versicolor*, the dialyzed nutrient solutions were used directly for the manometric measurements.

The ordinary manometric techniques described by Umbreit, Burris and Stauffer (1949) were used (cf. Lindeberg and Holm l.c. p. 103).

Results

Properties of the phenol oxidases secreted by Polyporus zonatus and P. versicolor

Several enzyme-containing solutions were obtained from media in which the fungi had been cultivated for two or three weeks. In order to characterize the enzymes, the specificity of the enzyme activity, the sensitivity towards carbon monoxide, and the influence of pH on the activity were studied. The results are to be found in Tables 1—2 and Figure 1.

Table 1 shows that the enzymes catalyze the oxidation of catechol, hydroquinone, p-phenylenediamine, and p-cresol. During the oxidation of the latter substance a white precipitate is formed. Tyrosine is not attacked. The enzyme activity is not inhibited by carbon monoxide (Table 2). Thus, the phenol oxidases of *P. zonatus* and *P. versicolor* are of the laccase type (cf. Lindeberg and Holm 1952).

Since in the presence of hydrogen peroxide, peroxidase oxidizes the same types of compounds as does laccase (cf Theorell 1951, p. 407), experiments were made to find out whether peroxidase was present in the solutions studied. The tests were made in Thunberg tubes which were filled with pure nitrogen. The reaction mixture contained pyrogallol, hydrogen peroxide, and the solution to be tested. No or a very weak peroxidase activity, as compared with the laccase activity, was observed.

Table 1. Activity of the phenol oxidases of *Polyporus zonatus* and *P. versicolor* in terms of $\mu\text{l O}_2$ uptake per hour at the oxidation of various substrates.

Substrate	Enzyme from <i>P. zonatus</i>	Enzyme from <i>P. versicolor</i>
Catechol	112	363
Hydroquinone	118	440
p-Phenylenediamine	94	310
p-Cresol	100	298
Tyrosine	0	0

Table 2. Influence of carbon monoxide on the enzyme activity.

Substrate and gas mixture	$\mu\text{l O}_2$ uptake per hour	
	Enzyme from <i>P. zonatus</i>	Enzyme from <i>P. versicolor</i>
Catechol, 90 0/0 N_2 + 10 0/0 O_2	134	41
» 90 0/0 CO + 10 0/0 O_2	128	43
p-Cresol, 90 0/0 N_2 + 10 0/0 O_2	124	—
» 90 0/0 CO + 10 0/0 O_2	120	—

The influence of the hydrogen ion concentration on the oxidation of catechol by the enzyme from *P. zonatus* is shown in Figure 1. A pronounced optimum was found at pH 5, the activity being definitely lower at pH 4 and 6. In a preliminary experiment, the maximum activity of the enzyme of *P. versicolor* was also found about pH 5. With respect to their dependence on the pH of the medium, the enzymes of *P. zonatus* and *P. versicolor* agree very much with the laccase secreted by the mycelium of the litter-decomposing species *Marasmius scorodonius* (Lindeberg and Holm l.c.).

Influence of some nutritional factors on the phenol oxidase formation

Preliminary experiments where different nutrient solutions were used indicated that the hydrogen ion concentration of the medium greatly influences the amounts of phenol oxidase secreted into the medium by the *Polyporus* species studied. The pH of the nutrient medium might be assumed to affect the final enzyme content of the medium in different ways:

- 1) by affecting the mycelial production of the fungus, the enzyme formation per unit mycelial weight remaining constant;
- 2) by affecting the secretion of the enzyme, e.g. the ratio intracellular/extracellular enzyme;
- 3) by affecting the intensity of enzyme formation, e.g. the amounts of enzyme formed per unit mycelial weight.

The influence of the hydrogen ion concentration of the medium on the phenol oxidase formation of the mycelia was studied in experiments, the results of which are to be found in Tables 3 and 4.

Each pH series comprised 20 parallel cultures. At the end of the experiment four mycelia from each series were used for the determination of the average dry weight. The mycelia and nutrient solutions of the remaining 16 flasks were poured into a graduated cylinder and the total volume was regulated to the original one by addition of distilled water. The solutions were filtered off and treated as follows below. The 16 mycelia from each series were rinsed with distilled water. Water was then added to a total volume of 60 ml, and to this 60 ml of a $1/5\text{-}M$ phosphate buffer,

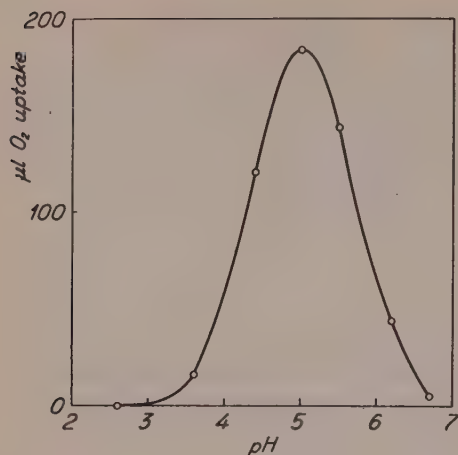


Fig. 1. Effect of pH on the activity of laccase from *Polyporus zonatus*. Substrate: catechol.

pH 6.8, were added. The mycelia were then ground in a »Turmix». After one hour the suspensions were centrifuged. 90 ml of the supernatants, corresponding to 12 mycelia, were saturated with ammonium sulphate. After 12 hours the precipitates were collected on »celite» filters and dialyzed against running tap water. The volume of the dialyzed solutions was regulated to 36 ml.

From each series 350 ml of filtered nutrient solution were treated as follows: The pH of the solutions was regulated to a value of 5—6 by addition of 0.1 N KOH. Thereafter the solutions were saturated with ammonium sulphate and the same procedure was followed as for the mycelial extracts. The volume of the dialyzed solutions was regulated to 28 ml by addition of distilled water.

The phenol oxidase activity of the dialyzed solutions was tested in the usual way, 2 ml extract and 1 ml $1/10$ -M citrate buffer being used in the main compartment of the Warburg vessels (pH 5.0). 2 ml of the mycelial extract corresponded to $2/3$ mycelium from one culture; 2 ml of the nutrient solution extracts corresponded to the solution from one culture.

Mycelia of *P. zonatus* were also extracted with buffer solution of pH 8.8 (borate buffer) and 2.8 (phosphate+hydrochloric acid). In no case did the mycelial extracts from this species show any oxidase activity. Likewise, the mycelium of *P. versicolor* showed very low or no intracellular phenol oxidase activity (cf. Fåhræus 1952, p. 288).

The influence of the hydrogen ion concentration on the phenol oxidase formation in *P. zonatus* was very pronounced (Table 3). The higher the hydrogen ion concentration, the greater was the enzyme production, totally as well as relatively. This effect was quite evident also within the pH-range where optimum growth occurred, e.g. between pH 4 and 6. At lower pH values, the effect rapidly increased. In the series of pH 2.6 the enzyme activity per mg mycelium formed was about 200 times greater than in the series of pH 5.

Table 3. *Influence of the hydrogen ion concentration of the medium on the amount of phenol oxidase secreted by Polyporus zonatus*. Incubation time 15 days. Enzyme activity expressed in terms of $\mu\text{l O}_2$ uptake in one hour at the oxidation of catechol.

Original pH	Final pH	Mycelial dry weight (mg)	$\mu\text{l O}_2$ uptake per culture	$\mu\text{l O}_2$ uptake per mg mycelium formed
2.6	2.6	4	329	82.3
3.1	3.0	34	287	8.4
4.0	4.0	63	89	1.4
4.9	5.1	66	25	0.4
5.8	6.0	62	18	0.3

The extract obtained from the solution of pH 3 was investigated with respect to substrate specificity and also with respect to the influence of carbon monoxide and hydrogen ion concentration on the enzyme activity. Catechol, hydroquinone, p-phenylenediamine, and p-cresol (white oxidation product) were oxidized, whereas tyrosine was not attacked. The enzyme activity towards catechol was insensitive to carbon monoxide and showed a pronounced maximum at pH 5. These results indicate that the enzyme secreted at pH 3 was identical with the one formed in the ordinary basic medium at pH 4.6.

In *Polyporus versicolor* (Table 4) the same phenomenon was observed though in this species the effect of pH on the laccase formation was less pronounced. The enzyme activity per mg mycelium was about 7 times greater in the pH 3.0 series than in that of pH 5.2. No growth was obtained with this species at pH 2.6.

In addition to the pH effect studied above, the influence on the laccase formation in *P. zonatus* of various inorganic salts was also tested. Whereas additions of potassium sulphate, sodium sulphate, or potassium nitrate (0.2—25 millimol/liter) had no effect, potassium iodide very strongly affected growth and enzyme production (Table 5). At concentrations from 1 to 25 millimol/liter, the development of the mycelium was completely inhibited. At

Table 4. *Influence of the hydrogen ion concentration of the medium on the amount of phenol oxidase secreted by Polyporus versicolor*. Incubation time 7 days. Enzyme activity expressed in terms of $\mu\text{l O}_2$ uptake in one hour at the oxidation of catechol.

Original pH	Final pH	Mycelial dry weight (mg)	$\mu\text{l O}_2$ uptake per culture	$\mu\text{l O}_2$ uptake per mg mycelium formed
3.0	3.1	6	48	8
3.6	3.7	14	56	4.0
4.2	4.2	19	80	4.2
5.2	4.7	36	40	1.1

Table 5. Influence of potassium iodide on the formation of phenol oxidase in *Polyporus zonatus*. Incubation time 18 days. Enzyme activity expressed in terms of $\mu\text{l O}_2$ uptake in one hour at the oxidation of catechol.

mg KJ per liter	Final pH	Mycelial dry weight (mg)	$\mu\text{l O}_2$ uptake per culture	$\mu\text{l O}_2$ uptake per mg mycelium formed
0	5.5	67	50	0.7
33	4.5	5	221	44

0.2 millimol/liter (33 mg/l.), however, a certain growth occurred. The laccase activity of the medium was strong. Thus, an addition of potassium iodide at a concentration which causes a decrease of the growth rate of the fungus, causes a marked increase of the laccase formation.

Conclusions

The phenol oxidases formed by the mycelia of *Polyporus zonatus* and *P. versicolor* are of the laccase type. In both species the enzyme is mainly extracellular in occurrence. However, the amount of enzyme secreted into the medium varies greatly with the composition of the latter. In the present paper mainly the influence of the hydrogen ion concentration has been studied. Increasing hydrogen ion concentration is followed by an increased secretion of the enzyme, caused by an intensified enzyme formation per unit mycelium, rather than by a changed permeability of the cell. This pH effect is evident within the pH range where optimum growth occurs, e.g. between pH 4 and 6, being greatly enhanced at supraoptimal hydrogen ion concentrations.

A similar pH effect on the formation of certain enzymes in fungi and bacteria has been shown earlier. In *Escherichia coli* and *Micrococcus lyso-deikticus* Gale and Epps (1942) observed an increased production of catalase, urease, fumarase, and certain dehydrogenases when the pH of the medium was lowered. Lindeberg and Malmgren (1952) found an increased formation of polymetaphosphatase in *Aspergillus niger* at low pH values.

It should be mentioned here that in the mycelia of various other hymenomycetes, e.g. species of the genera *Clavaria*, *Clitocybe*, *Lactarius*, *Lepiota*, *Marasmius* and *Psalliota*, laccase occurs both intra- and extracellularly (Lindeberg and Holm 1952). Preliminary experiments with *Marasmius graminum*, the laccase of which to a large extent occurs intracellularly, showed that supraoptimal hydrogen ion concentrations did not cause an increased laccase formation in this species. Thus, the question arises if the enzyme

formation in such species where the enzyme occurs only extracellularly, is more sensitive to certain factors of the surrounding medium than is the enzyme formation in species containing laccase of a more intracellular nature.

In this connection the influence of potassium iodide on the laccase formation in *Polyporus zonatus* is of certain interest. Evidently, an inhibition of growth, caused by quite another ion than the hydrogen ion, may be followed by an increased laccase production. In both cases an interference with the normal growth processes seems to be connected with increased laccase formation. Considering the similar action of laccase and of the system peroxidase+hydrogen peroxide, the latter also acting on iodide (cf. Theorell 1951, p. 407), the possibility should not, however, be excluded that laccase might, although very slowly, oxidize iodide and that the increased laccase formation is, in this case, adaptive in nature.

Summary

Polyporus zonatus and *P. versicolor* form phenol oxidases of the laccase type. In both species this enzyme occurs mainly extracellularly. Increasing hydrogen ion concentration of the medium causes an intensified laccase formation. A similar effect is obtained by adding potassium iodide to the medium at a concentration permitting only a slow growth of the mycelium (0.2 millimol KJ per liter).

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References

- Fåhræus, G.: Formation of laccase by *Polyporus versicolor* in different culture media. — *Physiol. Plant.* 5: 284. 1952.
- Gale, E. F. & Epps, H. M. R.: The effect of the pH of the medium during growth on the enzymic activities of bacteria (*Escherichia coli* and *Micrococcus lysodeikticus*) and the biological significance of the changes produced. — *Biochem. Journ.* 36: 600. 1942.
- Lindeberg, G. & Holm, G.: Occurrence of tyrosinase and laccase in fruit bodies and mycelia of some hymenomycetes. — *Physiol. Plant.* 5: 100. 1952.
- & Malmgren, H., Enzymatic breakdown of polymetaphosphate. IV. Influence of nutritional factors on the polymetaphosphatase production of *Aspergillus niger*. — *Acta Chem. Scand.* 6: 27. 1952.
- Theorell, H.: The iron-containing enzymes. B. Catalases and peroxidases. »Hydroperoxidases». — Sumner and Myrbäck: *The enzymes II*, 1: 397. 1951.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F.: *Manometric techniques and tissue metabolism*. — Burgess Publishing Co. Minneapolis. 1949.

Formation of Laccase by *Polyporus Versicolor* in Different Culture Media

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It has been shown repeatedly that numerous Basidiomycetes secrete a phenol oxidase from their mycelium when cultivated on ordinary laboratory media (for references, see 1, 7). Recent investigations have shown that, at least in many of these fungi, the phenol oxidase is of the laccase type (1, 2, 3, 5, 6, 7). It can now be considered firmly established that the *Polyporus* species, *P. versicolor* Fr. and *P. zonatus* Fr. form laccase but no tyrosinase, when grown in laboratory media.

Attempts were made to get solutions with a high laccase activity, and for that reason *Polyporus versicolor* was cultured in various media and for various periods of time.

Preliminary experiments showed that the enzymatic activity of the culture liquid was very dependent on the kind of medium used. While a conventional malt extract broth always showed a high laccase activity after 7 days incubation with *P. versicolor*, certain synthetic solutions were practically inactive after the same period, even though the rate of growth in the different solutions might have been the same. On the other hand, after a long incubation time the activity of the synthetic solutions was sometimes very high. Experiments were therefore made with *P. versicolor* in order to study these conditions more closely.

¹ A preliminary report of some of the data in this paper was given at the 7th Int. Bot. Congress, Stockholm 1950 (2).

Materials and Methods

A strain of *Polyporus versicolor* Fr. was used throughout this work. The culture was isolated from a fruitbody in 1945, and has already been used in earlier work (1).

The basal media had the following composition.

Medium M: Malt extract »Vitrum» 30 g, dist. water 1000 ml.

Medium A:

Glucose	20	g	FeSO ₄ · 7H ₂ O	0.01	g
Sodium acetate, 3H ₂ O	2.7	»	MnSO ₄ · 4H ₂ O	0.001	»
NH ₄ Cl	2.0	»	ZnSO ₄ · 7H ₂ O	0.001	»
KH ₂ PO ₄	0.5	»	CuSO ₄ · 5H ₂ O	0.0001	»
MgSO ₄ · 7H ₂ O	0.5	»	Aneurin	50	µg
CaCl ₂	0.01	»	Aq. dest.	1000	ml

Medium B: The same as A, except that 2.45 g L-asparagine was substituted for NH₄Cl and the acetate was omitted.

Conical flasks of 125 ml capacity containing 20 ml nutrient medium were inoculated with pieces of mycelium (4×4 mm) cut out from the mycelial mat on a malt extract agar plate. Care was taken that the pieces kept floating on the surface of the liquid.

At intervals, replicate flasks were withdrawn for analysis. The mycelia were filtered off, rinsed in water, dried at 100° C. and weighed. The filtrates were used for pH determination and for measurement of the enzymatic activity. This was done by means of the Warburg technique, the following amounts being used.

Main compartment 1 ml 0.1 M acetate buffer pH 5.0+1 ml enzyme solution; side arm 0.2 ml 0.1 M pyrogallol (in some experiments catechol); center well 0.2 ml 10 per cent KOH. Total volume 2.4 ml. The oxygen uptake was measured at 30° C.

The results of the manometric readings have been expressed as µl oxygen taken up in one hour per 1 ml of the original culture medium.

Sugar determinations were carried out in some experiments. The method of Bertrand was used.

Experiments and Results

Experiment 1. Two media were compared, viz. an ordinary malt extract broth (Medium M) and a synthetic medium (Medium A). Duplicate flasks were taken out every other day from 5 to 19 days, and after 28 days. The results are to be seen in the accompanying diagram (Figure 1). Each point represents the mean of the two samples, which were analyzed separately.

It can be seen that the two solutions have a rather different influence on the fungus. The malt extract seems to offer the best nutritive medium, and the growth curve is almost linear. In the synthetic solution, on the other hand, growth is somewhat delayed but after 11 days nearly equal to that in malt extract. About that time the mycelium shows signs of autolysis, which

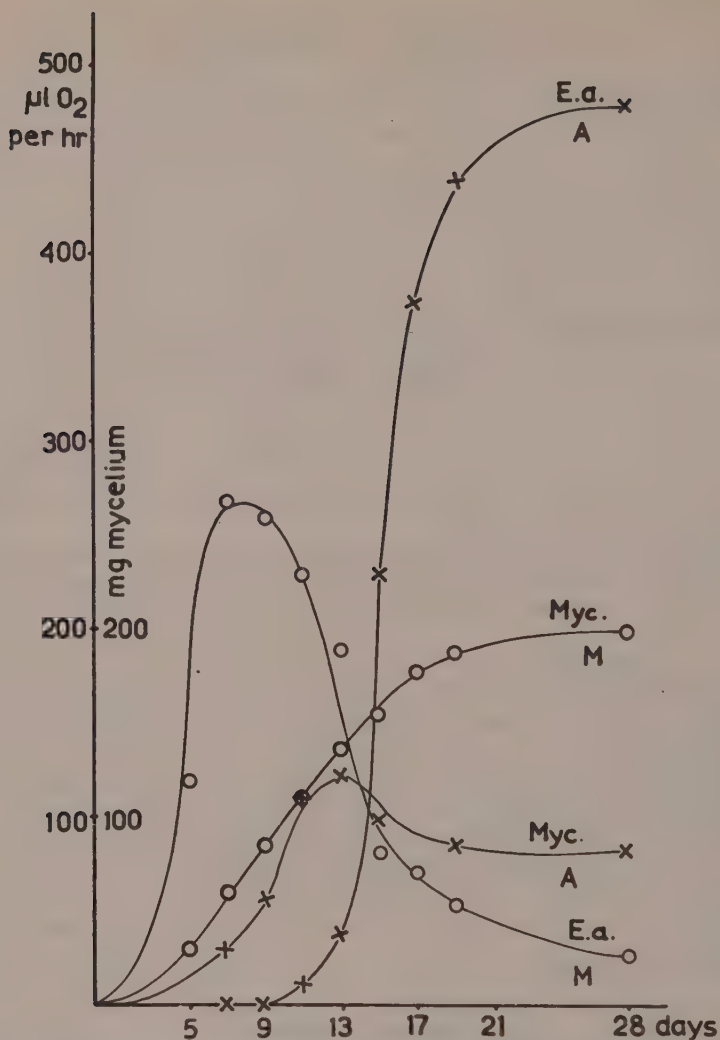


Figure 1. Formation of laccase (E.a.) and production of mycelium (Myc.) by *Polyporus versicolor* in two different media (M and A). The enzymatic activity is expressed as $\mu\text{l O}_2$ taken up per hour (Cf. p. 285).

again reduces the mycelial weight. Sugar determinations showed that at this point the glucose was practically exhausted. pH determinations revealed that at the time of maximal mycelial development in the A medium (c. 120 mg of dry weight) the pH had fallen to 2.4 but that later, due to the autolytic process, the neutral point was nearly reached. This is in contrast with the

Table 1. *Mycelial production and laccase formation of P. versicolor in two different media.*

Medium	Days	Mg mycelium per flask	Laccase activity per flask	Laccase activity per mg mycelium
M	7	59	5,320	90
	13	136	3,760	28
	19	186	760	4
A	7	29	0	0
	13	122	760	6
	19	84	9,500	78 ¹

¹ Calculated from the maximal weight of mycelium (122 mg).

malt extract medium, where only smaller changes in pH occurred during the growth.

The enzymatic activity of the two solutions also show interesting features. In the malt extract the laccase is secreted from the growing fungus almost immediately, whereas the synthetic solution shows very little, if any, activity in the early stages of development. This difference is most striking, if the large enzyme quantity secreted from 60 mg mycelium in malt extract (7 days: maximal enzyme quantity in this solution) is compared with the very small quantity secreted from 110 mg mycelium in the synthetic solution (11 days). This point is more clearly seen in Table 1.

From this Table and from Figure 1 it is also seen that the laccase activity very rapidly gets lost in the malt extract solution. If the activity is correlated with the actual mycelial weight, this loss is very pronounced, since a threefold increase in weight is accompanied by a steady decrease in enzymatic activity of the solution.

For a further discussion of the results, it is evidently important to know whether the phenol oxidase is perhaps formed by the fungus in both solutions but is secreted in only one of them, or whether it is not, or only to a very slight extent, formed by the mycelium growing in the synthetic solution. Since the increase in enzymatic activity of this latter solution runs about parallel with the autolysis of the mycelium, it would seem probable at first sight that the enzyme, previously formed, is released from the mycelium during this process.

However, this hypothesis was not supported by later findings. In a second experiment (Experiment 2) the enzyme formation was followed by analyzing the mycelium as well as the nutrient solution. The experiment was arranged in the same manner as Experiment 1, but this time medium B was also included.

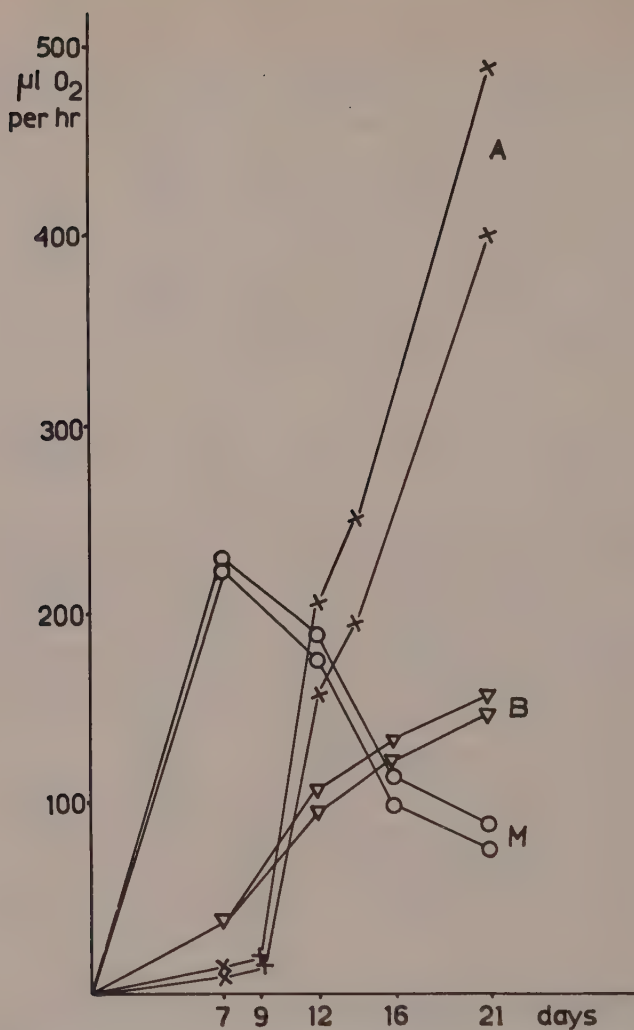


Figure 2. Formation of laccase by *Polyporus versicolor* in three different media (M, A and B). In each pair of curves the lower one represents the enzymatic activity ($\mu\text{l O}_2$ taken up per hour) of 1 ml of medium, the higher one the total activity (1 ml of medium + the corresponding amount of mycelium). The difference between the two parallel curves thus represents the laccase content of the mycelium.

4 flasks of each series were taken out for analysis at each sampling. The mycelia of two flasks were dried and weighed, and the other two flasks were used for measurement of the laccase activity. The solution was tested as usual; each mycelium was transferred to 3 ml $1/15$ M phosphate buffer of pH 6.8 and ground with fine sand in a mortar. The suspension was diluted to 10 ml and centrifuged. The super-

natant solution was then tested in manometric experiments after adjustment of pH to 5.0.

A similar extraction method was used successfully by Lindeberg and Holm (7) for the extraction of tyrosinase and laccase from the mycelium of different fungi.

The results of Experiment 2 will be seen from Figure 2.

The diagram shows that a very small part of the enzymatic activity is left in the mycelium. Only from the mycelia just undergoing autolysis (medium A after 14 and 21 days) could somewhat greater amounts of enzyme be extracted. In these cases, too, by far the largest part has been secreted into the medium.

In a later experiment it was also established that the residual mycelium after such an extraction had no phenol oxidase activity.

An objection which might be raised against the results given is that the enzyme activity was, as a rule, determined directly in the crude culture media. However, numerous control experiments have been carried out with the same media after dialysis in order to eliminate the possible influence of the low molecular-weight ingredients of the media. No difference in laccase activity was observed between the dialyzed and the non-dialyzed solutions.

Further experiments with *Polyporus versicolor* in various other media gave results in accordance with those mentioned above. Thus, natural extracts like yeast and potato extracts had a strong positive effect upon the formation of laccase in young cultures. On the other hand, synthetic media as a rule gave only a very slight activity after 1—2 weeks of incubation.

In addition, similar experiments have been performed with other wood-rotting fungi, *e.g.* *Polyporus zonatus* (3 strains), *P. hirsutus* (2 strains), *P. abietinus*, *Stereum hirsutum* (2 strains) and *S. purpureum*.

The *Polyporus* species showed a good activity and the course of laccase formation was essentially the same as in *P. versicolor* on corresponding media. The *Stereum* species, on the other hand, showed a very low activity in both A and M media, although the growth was fairly good, especially in the A medium. This might have some physiological significance; other experiments showed that the ability of the *Stereum* species to decompose lignin lies far behind that of the *Polyporus* species.

Discussion

It has been shown above that the production of laccase by *Polyporus versicolor* varies greatly with the composition of the growth medium. The two substrates mainly used in this work (M and A, *cfr.* p. 285) seem to represent rather extreme cases in this respect. In the first one the laccase

is soon formed and secreted into the medium, and in the other an insignificant amount of enzyme is formed by the growing mycelium, but when growth ceases and autolytic processes set in the laccase is formed in large quantities. The conditions under which the laccase is formed thus seem to be rather different in the two solutions, and it seems possible that quite different nutritional factors may affect the enzyme production. The following cases might be considered.

Some compound present in malt extract, for instance an amino acid or a vitamin, might promote the laccase formation, by directly or indirectly taking part in the synthesis of the enzyme molecule. From Figure 2 it seems possible to draw the conclusion that amino-N is more efficient than ammonium-N in stimulating the enzyme production. Some amino compounds may also be formed at a low rate by the fungus itself and thus cause a delayed formation of enzyme in the synthetic media.

Some constituent in the synthetic media, particularly medium A, may inhibit the formation of phenol oxidase.

Further, in medium A there occur certain changes during the course of the experiment, which may condition the enzyme production. These are a strong decrease in pH, which reaches values as low as 2.5, and the autolytic processes setting in at the exhaustion of the sugar, which again cause a strong increase in pH. In a previous communication (6) it was pointed out that the enzyme production in *P. zonatus* and to a certain extent also in *P. versicolor* was increased at low pH values. Similarly, when the A medium in the above experiments reaches pH values of 2.5—3, this may effect a stronger enzyme production.

In the autolytic process certain compounds (e.g., amino acids) are liberated which, as pointed out above, may promote the production of laccase. Some specific protein giving the active enzyme molecule under the influence of a proteinase might also be liberated. That autolytic processes may greatly increase the formation of other enzymes was shown recently by Lederberg (4).

The shortage of carbohydrate initiating the autolysis might directly influence the enzyme production. Finally, some oxidizable compound might effect an »adaptive» formation of phenol oxidase.

A question which also needs an answer is why the laccase is inactivated so rapidly in the malt extract medium. Since the activity of filtered media, also non-dialyzed ones, are maintained for a long time, the enzyme destruction seems to be a direct effect of the growing fungus. Possibly the reason simply is that proteinases are formed which are able to decompose the laccase.

Some of the questions raised in this discussion will be more closely analyzed in later communications.

Summary

Polyporus versicolor was cultured on different media and the formation of laccase was followed manometrically in Warburg experiments. It was found that in a non-synthetic substrate, like malt extract, considerable amounts of laccase were formed and secreted into the medium at an early stage of growth. In synthetic media the formation of laccase was delayed. A high activity was obtained in such media following autolysis of the mycelium. This was not connected with a release of preformed laccase in the mycelium, but the laccase was apparently formed during the autolysis process and directly secreted into the medium. Possible explanations of the findings are discussed.

As a result of the investigations it may be stated that, if active laccase solutions from fungi are desired, great attention must be paid to the composition of the medium and the period of incubation of the fungi used. In the present work, the highest activity was obtained by culturing the fungi for 21 days or more in a synthetic medium containing ammonium salt as the source of nitrogen and sodium acetate as a buffer substance.

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References

1. Fåhraeus, G.: On the oxidation of phenolic compounds by wood-rotting fungi. — Ann. Roy. Agr. Coll. Sweden 16: 618. 1949.
2. — Growth and excretion of phenol oxidase from the mycelium of *Polyporus versicolor*. — Proc. VII Int. Bot. Congr. Stockholm 1950.
3. Law, K.: Phenol oxidases in some wood-rotting fungi. — Ann. Bot. N. S. 14: 69. 1950.
4. Lederberg, J.: The beta-d-galactosidase of *Escherichia coli*, strain K-12. — Jour. Bact. 60: 381. 1950.
5. Lindeberg, G.: Phenol oxidases of the cultivated mushroom *Psalliota bispora* f. *albida*. — Nature 166: 739. 1950.
6. Lindeberg, G. & Fåhraeus, G.: Nature and formation of phenol oxidases in *Polyporus zonatus* and *P. versicolor*. — Physiol. Plant. 5: 277. 1952.
7. — & Holm, G.: Occurrence of tyrosinase and laccase in fruit bodies and mycelia of some hymenomycetes. — Physiol. Plant. 5: 100. 1952.

Inhibition of Respiration and Nitrate Absorption in Green Algae by Enzyme Poisons

By

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In some previous experiments (Österlind 1951), the author has shown that the respiration of *Scenedesmus quadricauda* is quite uninhibited even at very high concentrations of cyanide. The nitrate absorption, on the other hand, is totally inhibited at concentrations as low as 10 μ M HCN (added as KCN at pH 6.1—6.2). In the present investigation both *Scenedesmus quadricauda* and *Chlorella pyrenoidosa* have been used. In addition to cyanide, azide has also been used as an enzyme poison.

Influence of potassium cyanide on *Chlorella pyrenoidosa*

The respiratory measurements were performed in the vessels previously used (Österlind 1951). Cultivation and pretreatment of the algae have also been described in that paper. In Figure 1, the circles indicate the respiratory rates obtained during the first two hours in three experiments at pH 6.1 and in one experiment at pH 8.4. It has been possible to compare all four experiments by giving the experimental values in terms of percent of control. The fact that there seems to be no difference in the cyanide sensitivity of the alga at these two pH values must be due to the low value of the dissociation constant of HCN (K_a $7.2 \cdot 10^{-10}$, pK_a 9.14).

According to Fig. 1, the respiration of *Chlorella pyrenoidosa* is inhibited to 50 % at 50 μ M HCN, whereas, according

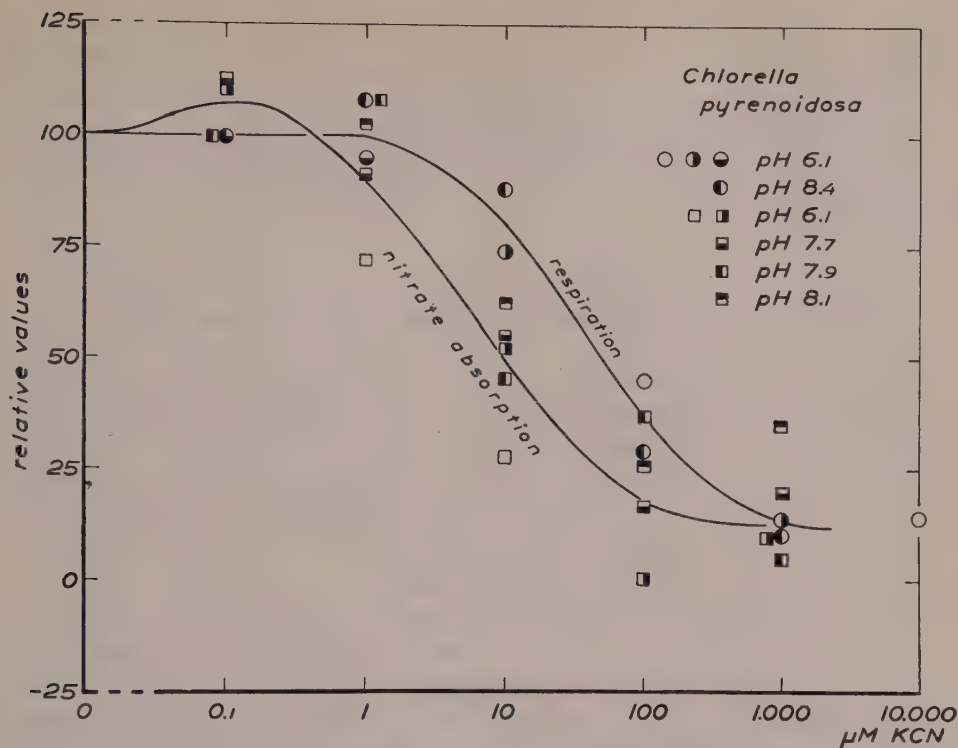


Figure 1. Respiration and nitrate absorption in *Chlorella pyrenoidosa* at various concentrations of cyanide.

to previous results, the respiration of *Scenedesmus quadricauda* was not inhibited at all by any concentration of HCN.

The measurements of nitrate absorption were performed at pH values between 6.1 and 8.1. The cells were pretreated as above but after centrifugation they were distributed to 100 ml conical flasks, each of which contained 40 ml of 0.02 M NaK-phosphate buffer of the desired pH. The dry-weight of the cells was about 100 mg per 100 ml solution and the concentration of nitrate at the beginning of the experiment about 0.180 mM. The flasks were shaken in the dark at 25°C for about 24 hours. The nitrate determinations were performed as previously. Maximal absorption in an experiment did not exceed 50 per cent of the initial content. The data from the determinations are given in Fig. 1, where the squares indicate the nitrate absorption in relative values of the absorption in the control vessel without cyanide. Each kind of square represents one experiment. As in the case of respiration, there seems to be no significant difference between the nitrate absorption at various pH values.

The nitrate absorption of *Chlorella pyrenoidosa* seems to be slightly more sensitive to HCN than is the respiration, 50 % inhibition being reached at 10 μ M HCN. Thus, the difference between the sensitivity of the two mechanisms to HCN is much smaller in the case of *Chlorella pyrenoidosa* than in *Scenedesmus quadricauda*.

Influence of sodium azide on *Scenedesmus quadricauda*

Determination of the respiratory rate at various concentrations of NaN_3 were made in four experiments at pH 4.7—5.2 (circles) and in two experiments at pH 8.1—8.5 (triangles) (Fig. 2). In the acid solutions, there is a slight stimulation of respiration at 10 μ M and 50 per cent inhibition at 100 μ M NaN_3 . In the alkaline solutions there is no stimulative or inhibitory effect of NaN_3 in the concentrations used. This must be due to the high value of the dissociation constant of HN_3 (K_a $1.9 \cdot 10^{-5}$, $\text{p}K_a$ 4.7), which means that at pH 4.7 half the total concentration of NaN_3 is undissociated HN_3 and half is N_3^- ions. At pH 5.0, about 3/10 of NaN_3 is in the form of HN_3 molecules and in the alkaline solutions used only about 2/10000 of the initial concentration constitutes uncharged molecules. The stimulative and inhibitory effect of NaN_3 on respiration has most likely been caused by the undissociated HN_3 molecules alone.

The measurement of nitrate absorption was performed at various pH values (the squares in Figure 2). Maximal absorption during an experiment was a little higher than in the above experiments with *Chlorella*, amounting to about 70 per cent of the initial nitrate content. Two experiments have been performed at pH 6.1—6.2, and one at each of the other two pH values. At 1000 μ M NaN_3 , it was impossible to make nitrate determinations with the phenol disulphonic acid method used, especially in alkaline solutions, since the values obtained were too low.

The algae are more sensitive to the azide at low pH values than at high ones. This is undoubtedly due to the fact that the algae are sensitive only to molecular HN_3 and not to N_3^- ions. At the pH values used, the concentration of HN_3 decreases about 10 times with every unit increase in pH. Therefore, if 50 per cent inhibition is caused by A μ M NaN_3 at pH B , 10 A μ M NaN_3 should be necessary to cause the same inhibition at pH $B+1$. This rule should hold for pH values above 5. Figure 1 shows that this rule agrees well with the experimental values.

At pH 5, 50 per cent inhibition of nitrate absorption is caused by 0.6 μ M NaN_3 , which corresponds to about 0.2 μ M HN_3 . At the same pH, however,

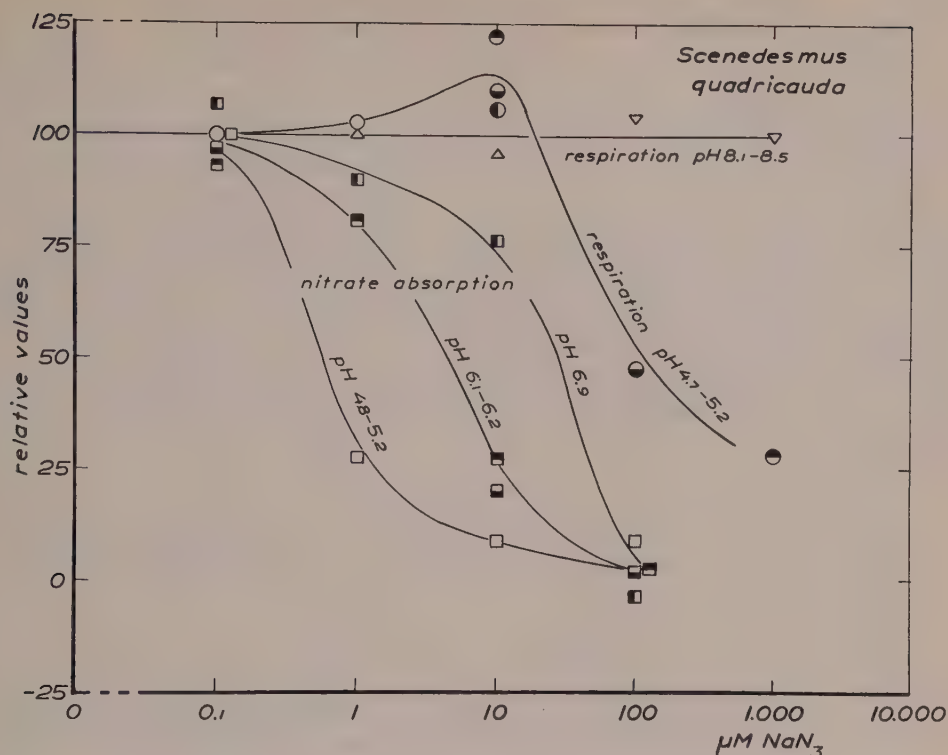


Figure 2. Respiration and nitrate absorption in *Scenedesmus quadricauda* at various concentrations of azide.

the respiration is inhibited to 50 per cent by 100 $\mu\text{M NaN}_3$, corresponding to about 30 $\mu\text{M HN}_3$ and a slight stimulation is found at 10 $\mu\text{M NaN}_3$, which corresponds to 3 $\mu\text{M HN}_3$. Thus, the nitrate absorption of *Scenedesmus quadricauda* is about 150 times more sensitive to azide than is the respiration.

Influence of sodium azide on *Chlorella pyrenoidosa*

The experimental data are shown in Figure 3. Only a rather low pH value was used. The stimulative and inhibitory effect on respiration and the inhibitory effect on nitrate absorption by NaN_3 on *Chlorella pyrenoidosa* agree with the results obtained with *Scenedesmus quadricauda* at the same pH.

Thus, the nitrate absorption of *Chlorella pyrenoidosa* is also about 150 times more sensitive to azide than is the respiration.

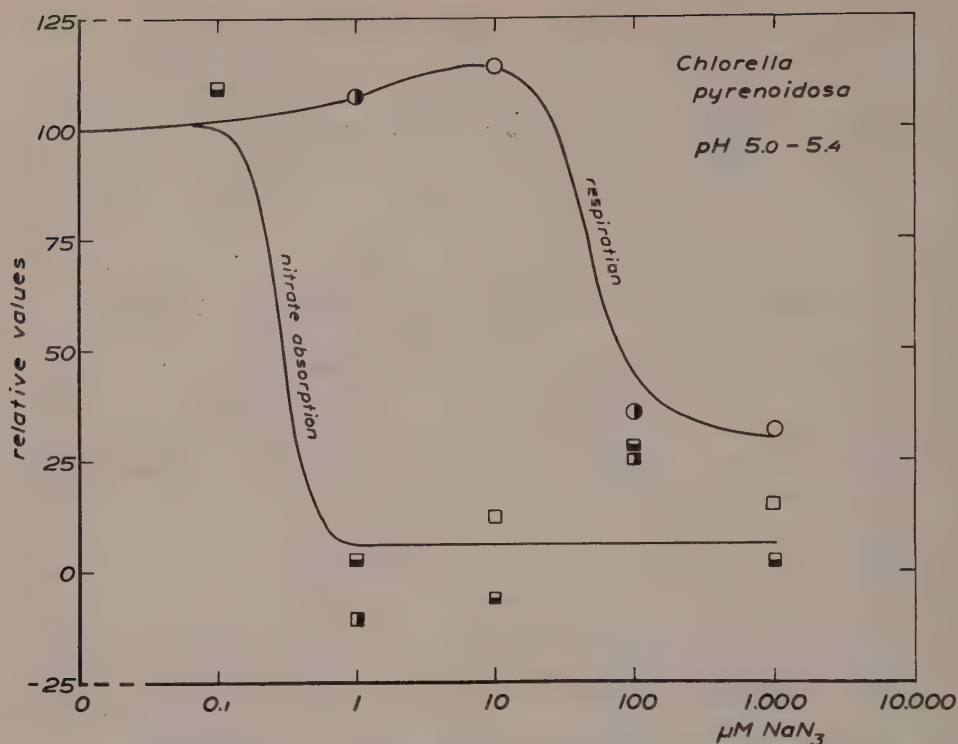


Figure 3. Respiration and nitrate absorption in *Chlorella pyrenoidosa* at various concentrations of azide.

Discussion

The unequal sensitivity of respiration and nitrate absorption in *Scenedesmus quadricauda* to cyanide has been discussed in a previous paper (Österlind 1951). The present experiments show that the unequal inhibition of the two processes is not restricted to cyanide and *Scenedesmus quadricauda* but also occurs with azide and with *Chlorella pyrenoidosa*. In one case (cyanide and *Chlorella pyrenoidosa*) the two processes are inhibited to about the same extent. This does not necessarily mean that the enzymes of the two processes are the same (they are differently inhibited by cyanide), but only that the two enzyme systems have the same sensitivity to a certain enzyme poison.

Summary

The effect of cyanide and azide on respiration and nitrate absorption in the green algae *Scenedesmus quadricauda* and *Chlorella pyrenoidosa* has been examined.

The respiration of *Scenedesmus* is not sensitive to cyanide (previous results), whereas the respiration of *Chlorella* is inhibited to 50 per cent by 50 μM HCN.

The sensitivity of nitrate absorption to cyanide is about the same in the two algae, *Scenedesmus* being inhibited to 50 per cent by 3 μM (previous results) and *Chlorella* by 10 μM .

Azide has the same influence on both *Scenedesmus* and *Chlorella*. Respiration is slightly stimulated by 3 μM HN_3 and inhibited to 50 per cent by 30 μM HN_3 . Nitrate absorption is inhibited to 50 % by 0.2 μM HN_3 .

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Reference

- Österlind, S.: Anion absorption by an alga with cyanide resistant respiration. — *Physiol. Plant.* 4: 528. 1951.

The Influence of Carbon Dioxide Concentration upon the Rate of Photosynthesis in *Sinapis alba*

By

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Introduction

The influence of carbon dioxide concentration upon the rate of photosynthesis in leaves has long been of considerable interest to plant physiologists. The earlier investigators were chiefly interested in determining optimal concentrations, and they found that assimilatory rate rose to a maximum and then declined with continued increase in carbon dioxide concentration, but it was not completely realized up to the time of Blackman (1905) that it is necessary to consider all the other factors affecting assimilation when the influence of one particular factor is to be studied. The presentation by Blackman of the concept of limiting factors emphasized this point and stimulated further research on the influence of various factors on photosynthesis, but there has been considerable disagreement with this concept by investigators who found no indication of the operation of single limiting factors in their studies of photosynthesis.

Boysen Jensen (1933) corrected determined rates of photosynthesis for the decrease of carbon dioxide concentration in the leaf chamber and expressed them as milligrams of carbon dioxide assimilated per 50 cm² per hour at a carbon dioxide concentration of 0.590 milligrams per liter, corresponding to 0.03 per cent carbon dioxide in the air at 0° C. and 760 mm. of mercury. The correction was based on the assumption that the rate of photosynthesis was directly proportional to the average carbon dioxide concentration in the leaf chamber. The aim of the present study was to check the correctness of this assumption and to establish the relationship between rate of photosynthesis and concentration of carbon dioxide around the normal 0.03 per

cent. The results, therefore, were not corrected for the decrease of carbon dioxide concentration in the leaf chamber but were plotted against the actual average concentration in the chamber.

Brown and Escombe (1902) concluded that photosynthesis in *Helianthus* leaves is approximately directly proportional to the amount of carbon dioxide present, but the conclusion was based on the use of only two concentrations. Blackman and Smith (1911) determined in experiments with *Elodea* and *Fontinalis* that at first the assimilation increased steadily in proportion to the increase of carbon dioxide supply and that this increase abruptly came to a stop, with *Elodea* at about 0.008 gram carbon dioxide per 100 ml. water and with *Fontinalis* at about 0.017 gram per 100 ml., in a manner similar to that of Blackman's ideal curve. Willstätter and Stoll (1918) studied the relation of assimilation to carbon dioxide concentration in *Helianthus* and *Urtica* to a slight extent, but only with concentrations of one volume per cent and above. Their data indicate a steady increase in photosynthetic rate up to the maximum 20 per cent used.

Warburg (1919), using 1/20 to 10 times the amount of carbon dioxide in equilibrium with the 0.03 per cent of the air, showed that in *Chlorella* the rate of assimilation steadily decreases with increasing carbon dioxide, contrary to the earlier findings, which he ascribes to the effects of the diffusion rate of carbon dioxide in the plant materials used. Lundegårdh (1921) obtained in *Nasturtium*, *Viola*, *Oxalis*, and *Melandrium* an approximately direct proportionality at lower concentrations, with a gradual falling off of rate with increasing carbon dioxide supply. From his work on cherry laurel Maskell (1928) concluded that he could confirm the direct proportionality reported by Brown and Escombe and by Lundegårdh.

James (1928) states that in *Fontinalis* there was no evidence that strict proportionality held over any range of the carbon dioxide concentrations that he used, but van den Honert (1930) employed films of *Hormidium* grown on the surfaces of nutrient solutions and determined that the assimilation curve was a straight line almost to the point of transition. van der Paauw (1932) obtained with *Hormidium* a curve in perfect agreement with that of van den Honert. Retardation of assimilation by phenyl urethane, however, resulted in the disappearance of the »Blackman type» curve and the formation of one which closely resembles the logarithmic curve Warburg found with *Chlorella*.

The first detailed studies on the effects of carbon dioxide concentrations in the region of the normal content of the air on higher land plants were carried out by Hoover, Johnston, and Brackett (1933). In experiments with whole plants of wheat they showed that the photosynthetic rate rises almost linearly with concentration, then passes through a transition range, and finally becomes independent of carbon dioxide concentration. In one experiment the proportionality persisted up to a concentration of about 0.035 volume per cent at the highest light intensity used.

Emerson and Arnold (1932) found in *Chlorella* that the assimilatory rate decreased steadily with increasing concentration in both continuous and intermittent light, and Emerson and Green (1934) derived similar results with the marine alga *Gigartina*. Smith (1936), however, working with *Cabomba*, obtained a curve which is at first linear and then gradually decreases in slope, and Singh and Lal (1935) present similar curves for sugar cane, wheat, and flax. Steemann Nielsen (1947) reported direct proportionality in *Myriophyllum* up to a concentration of about 0.009 grams carbon dioxide per liter of water, with maximal assimilation at about

three times that concentration at an optimal intensity of light. His curves for *Fontinalis* and *Cladophora* are similar but have sharper breaks.

Decker (1947) showed that the apparent photosynthetic rate of the entire shoots of apple tree seedlings varies linearly with the mean carbon dioxide concentration over the narrow range below the normal concentration of air that he studied and that the rate varies hyperbolically with the rate of air supply. Particular studies on assimilation in extremely low concentrations have been made by Miller and Burr (1935) and by Gabrielsen (1948). Gabrielsen has shown that assimilation in leaves of various higher plants does not take place at concentrations less than a threshold value of 0.009 volume per cent with an illumination of 10,000 Lux.

Thomas and Hill (1949) studied apparent assimilation by alfalfa, sugar beets, and tomatoes under field conditions in air enriched with carbon dioxide, and their results indicate a nearly linear increase in apparent assimilation with enrichment up to twelve times the content of normal air.

Materials and Methods

The present study of the relation of photosynthesis to carbon dioxide concentration in excised leaves of yellow mustard, was carried out with the use of the assimilation apparatus developed by Boysen Jensen (1928, 1932, 1933), with modifications described by Romose (1940). In this apparatus a stream of atmospheric air or of a mixture with desired carbon dioxide content is passed through a system which includes two small flattened glass tubes in series serving as leaf chambers. The air container is a spirometer with a capacity of 100 liters which is mounted in paraffin oil. At the beginning of an experiment the spirometer is filled with atmospheric air or with carbon dioxide-free air to which is added a desired quantity of pure carbon dioxide.

The air is passed from the spirometer to the leaf chambers through a U-tube containing strips of moist filter paper. The leaf chambers are kept in a bath of running water to maintain a constant temperature under the light source, which in the present case consisted of four 300-watt lamps, and the lamps are in addition cooled by running water. After passing through the leaf chambers the air is bubbled through an absorption tube containing Ba(OH)_2 . The air is drawn through the system by means of two aspirator bottles which provide a steady air stream and make possible an exact measurement of the amount of air drawn through the system.

The exact determination of the carbon dioxide content of the air entering and leaving the assimilation chambers is accomplished in the following manner: a known quantity of air is bubbled through a known quantity of $1/22$ normal Ba(OH)_2 , which absorbs the carbon dioxide. Untreated Ba(OH)_2 and the treated solution are then both titrated with HCl, and on the basis of the titrational difference, in milliliters of $1/110$ normal HCl, the exact carbon dioxide content of the air may be calculated.

Each of the individual experiments reported here lasted up to ten minutes, and in the intervals between them the air was passed over the leaves at the same rate as during the experiments. During such intervals the air was kept in motion by action of the spirometer, but it did not pass through the absorption tube. The Ba(OH)_2 solutions were removed from the tube during these periods, replaced with

fresh solutions, and then titrated. During the course of each experiment slightly more than two liters of air was drawn over the two experimental leaves, which together had a single-surface area of about 25 cm².

For the study young, well-developed leaves of *Sinapis alba* plants grown in the open in full sunlight were cut off, and the petiole of each leaf was immediately inserted into a small glass vase filled with water. The leaves were selected between nine and ten o'clock in the morning, and the extent of stomatal opening was checked on additional leaves by means of Boysen Jensen's stomatometer. The test leaves were checked at the conclusion of a series of experiments to make sure that the stomata had been open while the determinations were being made. During the experiments the illumination was at all times maintained at 23,000 Lux, and the temperature in the assimilatory chambers was kept at 20° C. The unpublished data of Müller and Bille Hansen (1949) which will also be presented were obtained subsequently using an illumination of 28,000 Lux at 20° C.

Results and Discussion

The apparent assimilation of the *Sinapis* leaves in milligrams of carbon dioxide absorbed per 50 cm.² of upper leaf surface per hour plotted against the carbon dioxide concentration of the air is presented in Figure 1. A logarithmic relationship of the points determined is indicated, and plotting of the assimilation against the logarithm of the concentration in milligrams per 10 liters of air does produce a straight-line curve for the concentrations studied. From the regression formula of this ($\bar{Y}=14.14X-1.02$) the curve in Figure 1 is derived. The concentrations range from 0.219 to 1.784 milligrams per liter, i.e., from about one-third of the normal 0.59 milligrams per liter of the air to three times the normal content.

Unpublished data of Müller and Bille Hansen (1949) for portions of the same range with *Sinapis* are included with the above results. As has been mentioned, their data were secured at a light intensity of 28,000 Lux, while the present study was carried out at an intensity of 23,000 Lux. The comparability of the data indicates that an intensity of 23,000 Lux was optimal.

The assimilation reported here is apparent assimilation, since a correction is not made for the carbon dioxide released by respiration of the leaves. The production of carbon dioxide by respiratory activity, however, is small compared to the maximum amount assimilated (Boysen Jensen (1932) reports a value of 0.87 milligrams per 50 cm.² per hour for *Sinapis* in normal air at 20° C.) and is assumed to remain constant in rate under the present conditions. The carbon dioxide concentration of the air in the assimilatory chambers was taken to be the mean of the concentrations of the air entering and the air leaving the chambers. Calculations were based on saturated air at prevailing atmospheric pressures.

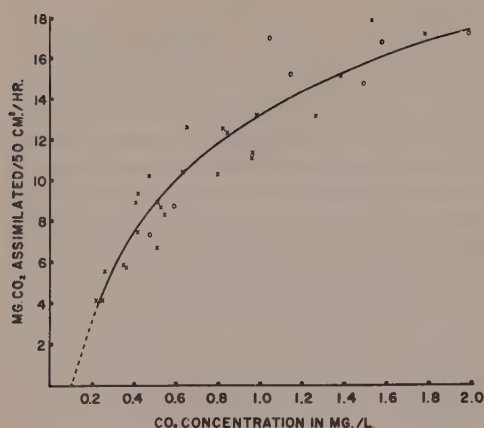


Figure 1. Relation of apparent assimilation to carbon dioxide concentration in excised leaves of *Sinapis alba*. The circles represent data of Müller and Bille Hansen (1949).

As would be expected from the work on the carbon dioxide threshold of leaves of higher plants, the assimilation curve does not pass through the origin but strikes the abscissa at about 0.11 milligrams per liter, the point found by Gabrielsen (1948) for *Sambucus* being at 0.17 milligrams per liter (0.009 per cent). If the logarithmic relationship holds below the range of carbon dioxide concentrations actually tested, it should be possible by extrapolation of the line to estimate the concentration at which there will be equilibrium between photosynthesis and respiration at optimal illumination.

The apparent assimilatory rate of 9.5 milligrams carbon dioxide per 50 cm² per hour corresponding to 0.55 milligrams per liter air (near the normal concentration of air) is slightly lower than the ca. 10.9 milligrams per hour reported for *Sinapis* by Boysen Jensen (1933) with the same conditions.

The conclusion that assimilation in *Sinapis* decreases progressively in rate with increasing carbon dioxide concentration is in agreement with work on the lower water plants *Chlorella* (Warburg, 1919; Emerson and Arnold, 1932), *Gigartina* (Emerson and Green, 1934), and *Fontinalis* (James, 1928), but is at variance with the direct proportionality at lower concentrations reported in higher land plants by Brown and Escombe (1902), Willstätter and Stoll (1918), Lundegårdh (1921), Maskell (1928), Hoover et al. (1933), Singh and Lal (1935), Decker (1947), and Thomas and Hill (1949). Smith (1936) suggested as a criterion for the validity of any theoretical description of photosynthesis the equation $KC = p/(p_{\max} - p)^{1/2}$ where p is the rate of photosynthesis at carbon dioxide concentration C , K is a constant, and p_{\max} is the asymptotic maximum rate of photosynthesis. The data presented here fit this equation fairly well, assuming a maximum rate of about 22 milligrams carbon dioxide absorbed per 50 cm² per hour.

Conclusions

Assimilation of carbon dioxide by excised leaves of *Sinapis alba* under conditions of constant optimal illumination and constant temperature is a logarithmic function of the carbon dioxide concentration in the surrounding air in concentrations up to three times that of ordinary air. The rate of photosynthesis at normal carbon dioxide concentration with optimal illumination is at least partially limited by insufficient carbon dioxide supply.

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References

1. Blackman, F. F.: Optima and limiting factors. — *Ann. Bot.* 19: 281. 1905.
2. Blackman, F. F., and A. Smith: Experimental researches on vegetable assimilation and respiration. IX. On assimilation in submerged water-plants, and its relation to the concentration of carbon dioxide and other factors. — *Proc. Roy. Soc. (B)* 83: 389. 1911.
3. Boysen Jensen, P.: Über neue Apparate zur Messung der Kohlensäureassimilation, der Respiration, der Öffnungsweite der Spaltöffnungen und der Beleuchtungsstärke. — *Planta* 6: 456. 1928.
4. — Die Stoffproduktion der Pflanzen. Jena. 1932.
5. — Über die Bestimmung der Assimilationsintensität. — *Planta* 21: 368. 1933.
6. Brown, H. T., and F. Escombe: The influence of varying amounts of carbon dioxide in the air on the photosynthetic process of leaves and on the mode of growth of plants. — *Proc. Roy. Soc. (B)* 70: 397. 1902.
7. Decker, J. P.: The effect of air supply on apparent photosynthesis. — *Pl. Physiol.* 22: 561. 1947.
8. Emerson, R., and W. Arnold: A separation of the reactions in photosynthesis by means of intermittent light. — *Jour. Gen. Physiol.* 15: 391. 1932.
9. Emerson, R., and L. Green: Manometric measurements of photosynthesis in the marine alga *Gigartina*. — *Jour. Gen. Physiol.* 17: 817. 1934.
10. Gabrielsen, E. K.: Threshold value of carbon dioxide concentration in photosynthesis of foliage leaves. — *Nature* 161: 138. 1948.
11. Hoover, W. H., E. S. Johnston, and F. S. Brackett: Carbon dioxide assimilation in a higher plant. — *Smithsonian Misc. Coll.* 87, No. 16: 1. 1933.
12. James, W. O.: Experimental researches on vegetable assimilation and respiration. XIX. The effect of variations of carbon dioxide supply upon the rate of assimilation of submerged water plants. — *Proc. Roy. Soc. (B)* 103: 1. 1928.
13. Lundegårdh, H.: Ecological studies in the assimilation of certain forest-plants and shore-plants. — *Sv. Bot. Tidskr.* 15: 46. 1921.
14. Maskell, E. J.: Experimental researches on vegetable assimilation and respiration. XVIII. The relation between stomatal opening and assimilation. A critical study

- of assimilation rates and porometer rates in leaves of cherry laurel. — Proc. Roy. Soc. (B) 102:488. 1928.
15. Miller, E. S., and G. O. Burr: Carbon dioxide balance at high light intensities. — Pl. Physiol. 10: 93. 1935.
 16. Müller, D., and E. Bille Hansen: Unpublished data. 1949.
 17. Romose, V.: Ökologische Untersuchungen über *Homalothecium sericeum*, seine Wachstumsperioden und seine Stoffproduktion. — Dansk bot. Ark. 10, No. 4: 1. 1940.
 18. Singh, B., and K. Lal: Limitations of Blackman's law of limiting factors and Harder's concept of relative minimum as applied to photosynthesis. — Pl. Physiol. 10: 245. 1935.
 19. Smith, E. L.: Photosynthesis in relation to light and carbon dioxide. — Proc. Nat. Acad. Sci. 22: 504. 1936.
 20. Steemann Nielsen, E.: Photosynthesis of aquatic plants with special reference to the carbon sources. — Dansk bot. Ark. 12, No. 8: 1. 1947.
 21. Thomas, M. D., and G. R. Hill: Photosynthesis under field conditions. In *Photosynthesis in Plants*, ed. J. Franck and W. E. Loomis, Ames, Iowa. 1949.
 22. van den Honert, T. H.: Carbon dioxide assimilation and limiting factors. — Rec. trav. bot. néerl. 27: 149. 1930.
 23. van der Paauw, F.: The indirect action of external factors in photosynthesis. — Rec. trav. bot. néerl. 29: 497. 1932.
 24. Warburg, O.: Über die Geschwindigkeit der photochemischen Kohlensäurezersetzung in lebenden Zellen. — Biochem. Zeitschr. 100: 230. 1919.
 25. Willstätter, R., and A. Stoll: Untersuchungen über die Assimilation der Kohlensäure. Berlin. 1918.

On the Effects of Weak Auxins and Antiauxins upon Root Growth

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Introduction

Among the synthetic growth substances the unsubstituted phenyl- and cyclohexyl-alkylcarboxylic acids are generally found to have weaker effects than the corresponding indolyl or naphthyl derivatives (17, 27, 32, 33). Some of them, e.g. phenylbutyric acid (PB) and cyclohexylacetic acid (CHA), have even been thought to lack true auxin activity completely, being active in a preparatory reaction only (33). Also the unsubstituted phenoxy acids can seemingly be added to the group of weak auxins, being much less active than the corresponding 2-naphthoxy derivatives at least.

Starting from the supposition that the ring system, or nucleus, is one of the main factors effecting the binding of the growth substance molecules to the active positions within the plasma, it seemed worth while to study the effect of an auxin antagonist upon the activity of these weak auxins in order to find out if they are perhaps more loosely bound and easier to replace than the typical auxins. Such a weak adsorption power could then be the common basis of the generally low activity of the growth substances with unsubstituted phenyl or cyclohexyl nuclei.

The following substances have been used in this study:

PA: phenyl-acetic acid, $C_6H_5 \cdot CH_2 \cdot COOH$, (puriss.).

PB: γ -phenyl-butyric acid, $C_6H_5 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$, (Eastman Kodak Co.).

CHA: cyclohexyl-acetic acid, $C_6H_{11} \cdot CH_2 \cdot COOH$, (a purified preparation

obtained by the courtesy of Prof. N. Hellström, Uppsala; recrystallized several times from formic acid).

POA: phenoxy-acetic acid, $C_6H_5 \cdot O \cdot CH_2 \cdot COOH$, (PAL Chemicals Lt., London).

POP: α -phenoxy-propionic acid, $C_6H_5 \cdot O \cdot CH(CH_3) \cdot COOH$. Of this acid the racemic mixture (*r*) as well as the dextro-rotatory (+) and the laevo-rotatory (—) forms were used. They were obtained by the courtesy of Prof. A. Fredga (see 12).

POB: α -phenoxy-iso-butyric acid, $C_6H_5 \cdot O \cdot C(CH_3)_2 \cdot COOH$, (melting point $100^\circ C$; obtained by the courtesy of Fil. lic. M. Matell).

IA: 3-indolyl-acetic acid, $C_8H_6N \cdot CH_2 \cdot COOH$.

2,4-D: 2,4-dichlorophenoxy-acetic acid, $C_6H_3Cl_2 \cdot O \cdot CH_2 \cdot COOH$.

1-NA: 1-naphthyl-acetic acid, $C_{10}H_7 \cdot CH_2 \cdot COOH$.

1-NMSP: α -(1-naphthylmethyl-sulfide)-propionic acid, $C_{10}H_7 \cdot CH_2 \cdot S \cdot CH(CH_3) \cdot COOH$, (obtained by the courtesy of Prof. A. Fredga, see 10).

All tests were made with young flax seedlings, growing in solution culture at $25^\circ C$. The solutions contained a phosphate buffer (6.7 mM), 5 mM Ca-nitrate and the substance to be tested; the pH was maintained as close to 5.9 as possible. The root growth was measured after a test period of 17 hours. The test is identical with the S-test described in an earlier communication (1) where further details can be found.

Effects of PA, PB and CHA

The action curves of the pure substances are shown in figure 1, where the 2,4-D and IA curves are also plotted for the sake of comparison. The much lower activity of the phenyl and cyclohexyl compounds is also apparent from table 1, where some data for other roots are included. The values for 50 per cent inhibition are not sufficient, however, to characterize the effects of the substances, as the slope of the curves is also different. The increase in concentration needed to augment the inhibition from 20 to 80 per cent is $28 \times$ for IA, and $35 \times$ for CHA, but only 6—8 \times for 2,4-D, PA and PB. This difference is also apparent in the data for IA and PA presented by Moewus (19) and by Bonner & Koepfli (4).

It is quite possible that the differences in slope, and also the stimulations occurring at low concentrations in the *Lepidium* test, are related to adaptation phenomena. For IA at least there is a fairly rapid recovery in the initially depressed growth rate at low concentrations (4, 5, 6, 18), and it is thus to be expected that the form of the action curve will vary with the

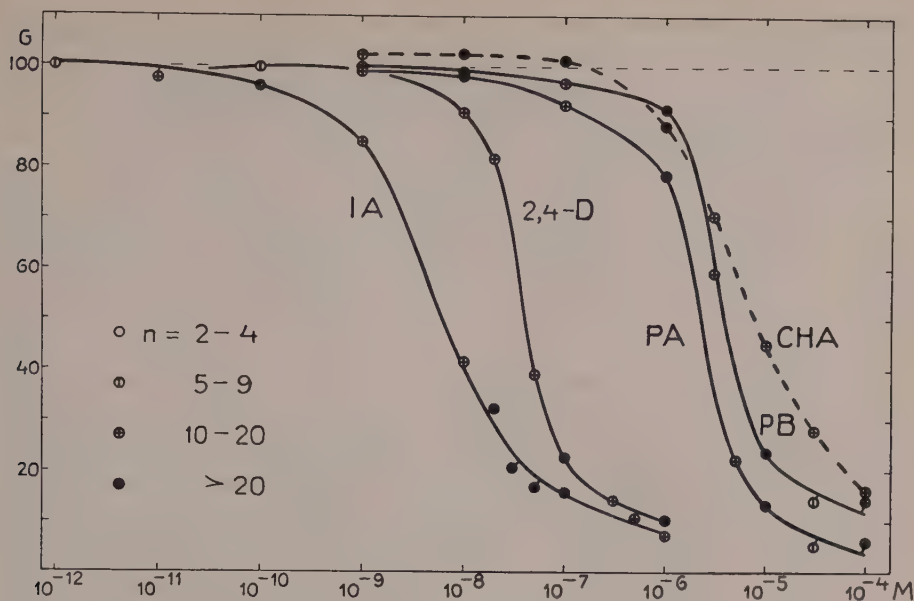


Fig. 1. Effects of strong (IA, 2,4-D) and weak auxins (PA, PB, CHA) on the root growth of flax seedlings. Root growth in per cent of control (G) plotted against a logarithmic concentration scale (M = mole per litre); n is the number of tubes, each with 14 seedlings, represented by the points.

length of the test period. The stimulations reported for *Lepidium* roots (19, 20, 9) occur in the range of 10^{-9} – 10^{-11} M IA or 10^{-7} – 10^{-8} M PA; no consistent stimulations by low concentrations of these substances were found by Bonner & Koepfli (4) for *Avena* roots, nor could they be detected for *Linum* roots in the present investigation. Further studies are necessary to find out if the adaptation process is perhaps more rapid in the *Lepidium* roots, or if the stimulation is possibly due to other causes inherent in the test method used.

PA, PB and CHA were further tested in combination with 10^{-7} M 2,4-D (tables 2–4); the average effects have been included in table 8. For PA the effects seem to be clearly additive. 10^{-6} M PA is equivalent to 0.23×10^{-7} M 2,4-D, and 10^{-5} M PA to 6.3×10^{-7} M 2,4-D, as estimated from the standard curves of fig. 1. Now the growth in 1.23×10^{-7} or in 7.3×10^{-7} M 2,4-D, expressed as per cent of that in 10^{-7} M 2,4-D, is 88 or 49 respectively, which numbers are in good agreement with those of table 8. For the mixture 10^{-7} M 2,4-D + 10^{-6} M CHA we calculate a G' -value of 92 which compares well with the observed value of 96. For the PB-mixtures, however, this

Table 1. *Molar concentrations needed for 50 per cent inhibition of root growth. Data for Avena from Bonner & Koepfli (4), for Lepidium from Moewus (19, 20).*

Substance	Soln. culture	On moistened filter paper		
	<i>Linum</i>	<i>Linum</i>	<i>Avena</i>	<i>Lepidium</i>
IA	0.6×10^{-8}	2.5×10^{-7}	3×10^{-7}	2.4×10^{-7}
2,4-D	3.9×10^{-8}	7.1×10^{-7}	—	—
1-NA	6.3×10^{-8}	10.6×10^{-7}	70×10^{-7}	—
PA	2.2×10^{-6}	—	10×10^{-5}	7.6×10^{-5}
PB	3.8×10^{-6}	—	100×10^{-5}	—
CHA	7.6×10^{-6}	—	100×10^{-5}	—

Table 2. *Root growth in PA with additions of 1-NMSP or 2,4-D. C = the growth of control roots in mm/17 h. n = number of tubes, each with 14 seedlings, used for each concentration (molarity denoted by an exponential number).*

Exp.	C	n	Root growth, relative values (C = 100)				
			5×10^{-6} PA + 1-NMSP:				
			0	10^{-7}	10^{-6}	5×10^{-6}	10^{-5}
1	21.2	4	21.6	23.9	35.6	63.0	75.4
2	21.4	4	26.2	30.4	29.7	52.1	69.6
3	23.1	4	23.3	24.6	33.9	58.5	68.5
4	22.4	4	18.2	21.4	35.0	65.6	68.1
			10^{-7} 2,4-D + PA:			PA:	
			0	10^{-6}	10^{-5}	10^{-6}	10^{-5}
5	20.8	4	19.2	17.1	11.4	81.2	14.2
6	21.6	4	23.2	20.4	13.5	87.8	15.0

Table 3. *Root growth in PB with additions of 2,4-D or 1-NMSP. Values presented as in table 2.*

Exp.	C	n	Root growth, relative values (C = 100)						
			10^{-7} 2,4-D + PB:			PB:		10^{-5} PB + 1-NMSP:	
			0	10^{-6}	10^{-5}	10^{-6}	10^{-5}	10^{-5}	2×10^{-5}
7	26.2	5	22.5	22.6	—	95.0	—	—	—
8	27.2	5	23.2	28.6	—	94.9	—	—	—
9	18.8	4	20.0	20.8	22.5	87.2	28.6	—	—
10	21.9	4	19.0	19.8	19.2	90.9	26.9	—	—
11	19.9	4	23.9	22.0	22.8	97.2	30.3	—	—
12	25.0	4	21.0	18.7	—	86.7	19.6	39.1	—
13	24.9	4	20.8	18.5	—	83.5	20.3	33.5	—
14	25.0	4	—	—	—	—	18.6	31.8	40.2
15	24.9	4	—	—	—	—	21.3	35.8	43.0

Table 4. Root growth in CHA with additions of 2,4-D or 1-NMSP. Values presented as in table 2.

Exp.	C	n	Root growth, relative values (C = 100)				
			10 ⁻⁷ 2,4-D + CHA:		CHA	10 ⁻⁵ CHA + 1-NMSP:	
			0	10 ⁻⁶	10 ⁻⁶	0	10 ⁻⁵
16	26.2	5	22.5	22.7	89.7	—	—
17	27.2	5	23.2	23.7	89.6	—	—
18	26.5	4	20.5	19.3	89.0	46.0	69.2
19	26.7	4	19.3	16.9	87.2	40.6	61.6

method of calculation is apparently not feasible, PB being more likely a weak 2,4-D antagonist in the concentration range tested.

When, on the other hand, an auxin antagonist like 1-NMSP (1, 3) is added to PA, PB or CHA, the growth inhibition caused by these substances alone is strongly counteracted (tables 2—4). The average effects of the 1-NMSP additions are presented in table 5. The »activities» of PA, PB or CHA when combined with 1-NMSP have been evaluated from the curves of fig. 1, and after division by the actual concentration (5×10^{-6} M for PA, 10^{-5} M for PB and CHA) the activity coefficients A are obtained (cf. 1, 3). From table 5 it is apparent that an equimolar concentration of 1-NMSP will lower the activity of PA to 0.37, of PB to 0.57, and of CHA to 0.38, while the activity of 2,4-D is lowered only to 0.79. It will thus seem that the effect

Table 5. The effects of 1-NMSP upon the action of 2,4-D and some weak auxins.

1-NMSP M	2,4-D 10 ⁻⁷	(+)-POP 3×10^{-6}	PA 5×10^{-6}	PB 10 ⁻⁵	CHA 10 ⁻⁵
0	Root growth, rel. values (C = 100)				
	22.6	18.3	22.3	23.7	45.0
	Do. (growth in pure auxin = 100)				
10 ⁻⁷	120	—	113	—	—
10 ⁻⁶	140	—	154	—	—
3×10^{-6}	210	—	—	—	—
5×10^{-6}	—	—	276	—	—
10 ⁻⁵	301	367	321	176	151
2×10^{-5}	379	—	—	209	—
	Activity coefficients (A)				
	10 ⁻⁷	0.79	—	0.89	—
	10 ⁻⁶	0.65	—	0.65	—
	3×10^{-6}	0.46	—	—	—
	5×10^{-6}	—	0.37	—	—
	10 ⁻⁵	0.30	0.29	0.28	0.57
	2×10^{-5}	0.18	—	—	0.47
					—

(the competitive action) of 1-NMSP upon PA, PB or CHA is much stronger than its effect upon 2,4-D. This fact would apparently be in accord with the hypothesis that the comparatively low growth substance activity of the phenylic or cyclohexylic substances is due mainly, or to some extent at least, to their low affinity for the active positions within the plasm. It must be remembered, however, that the comparison of the effects of equimolar concentrations of 1-NMSP upon the different substances must be made at different absolute concentrations, and that complications are liable to arise from this circumstance.

Comparing the effects of 1-NMSP on PA and PB we find it very probable that PA is easier to replace than PB. Now, PA is the more active substance in repressing root growth, and thus we are compelled to assume a conspicuously higher activity of the *bound* form of PA compared to that of PB, an assumption which also gives a clue to the explanation of their differential behaviour in combination with 2,4-D (p. 314).

Effects of some phenoxy-alkylcarboxylic acids

The action curves of POA, POP and POB are presented in figure 2. Of POP the two optically active forms as well as the racemic mixture have been tested. Apparently (+)-POP is the only one of this group of substances which has an activity comparable to that of other weak auxins with unsubstituted phenyl nuclei. The molar concentration needed for 50 % inhibition of root growth is 1.3×10^{-6} and compares well with the corresponding value for PA (2.2×10^{-6}). The inhibitions caused by POA and POB appear at very high concentrations (10^{-4} — 10^{-3} M), and are most likely unspecific toxic effects comparable to those observed for various other auxin antagonists (3, 8).

The racemic POP has about half the activity of the (+)-form, indicating a total lack of activity for (—)-POP. In combination experiments with 2,4-D (table 6) no appreciable antagonistic effect was noted, and with (+)-POP the effect seems to be additive. The inhibiting effect of (—)-POP is about 1 per cent of that of (+)-POP. As a contamination of this magnitude is hardly possible (12), it seems reasonable to conclude that the change in configuration has, in this case, not been sufficient to eliminate the auxin activity of the molecule altogether.

That the effects of (+)-POP upon root growth are of the same nature as those of e.g. PA is made highly probable by the combination experiments presented in table 6, which shows effects of the same type as does table 2. 1-NMSP could be used in a concentration 3.3 times higher than that of

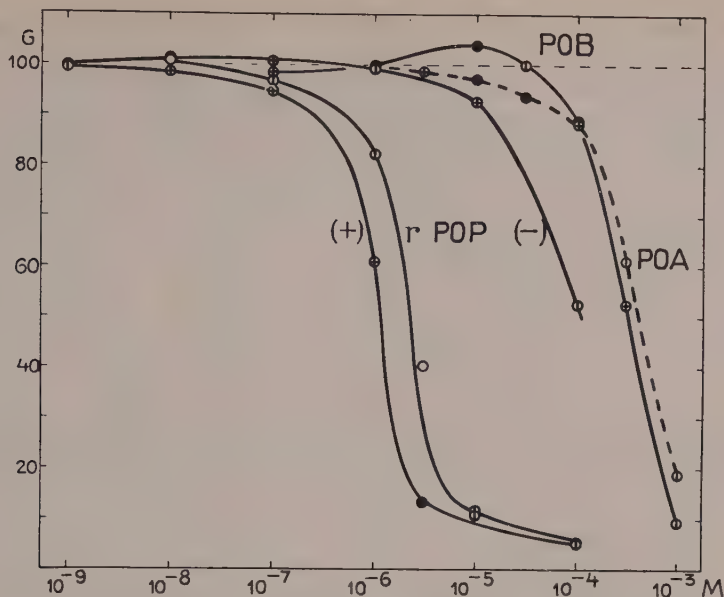


Fig. 2. Effects of some phenoxy acids on root growth. Values presented as in fig. 1. G-values for low POA concentrations: 10^{-8} 99.5 ($n=24$), 10^{-7} 101.4 ($n=12$), 10^{-6} 101.4 ($n=8$).

(+)-POP, and, with due regard to this fact, the magnitude of the antagonistic effect seems to be well comparable to that exerted upon PA or CHA (table 5). When slightly inhibiting concentrations of 2,4-D and (+)-POP are combined, the effects of the two substances are probably added.

That the racemic POP, contrary to the inactive POA, possesses several types of growth substance effects has been repeatedly noted. Thus, it exerts a weak root-inducing activity (15, 16), causes parthenocarpic development of tomatoes (34) and apples (23), modifies the leaf shape and causes epinastic bendings (34, 35, 22). It is noteworthy that α -phenoxy-*n*-butyric acid seems to have effects which come very near to those of POP.

The effects of POA, racemic POP, and POB upon the growth of wheat roots have recently been studied by Burström (7, 8) who used a test period of 3 days. He finds POA practically growth inactive (a slight stimulation of questionable significance can be noted at 10^{-5} M), POP strongly growth retarding (69 per cent inhibition at 10^{-6} M), while POB gives very conspicuous growth stimulations (growth doubled or more at 10^{-5} M) and is also shown to counteract the effects of POP. The growth stimulations obtained by Burström for wheat roots are apparently much stronger than those found for flax roots in the present study. The average stimulation

Table 6. *The interactions of (+)-POP, (—)-POP, 2,4-D and 1-NMSP. Values presented as in table 2.*

Exp.	C	n	Root growth, relative values (C = 100)				
			3×10^{-6} (+)-POP + 1-NMSP:		10^{-8} 2,4-D + (+)-POP:		(+)-POP
			0	10^{-5}	0	10^{-7}	10^{-7}
20	26.5	4	18.9	68.4	—	—	—
21	27.5	4	16.9	66.9	91.3	85.0	92.9
22	26.2	4	18.9	64.8	94.3	88.5	95.1
23	28.8	4	—	—	90.1	91.1	91.8
			10^{-7} 2,4-D + (—)-POP:		3×10^{-6} (+)-POP + (—)-POP:		(—)-POP
			0	10^{-5}	0	10^{-5}	10^{-5}
24	28.6	4	22.7	22.4	17.5	16.3	89.9
25	27.5	4	21.1	21.4	17.4	15.8	94.1

in 10^{-5} M POB is thus only 4 per cent, but seems to be significant (rel. growth value 104.0 ± 0.99); for POA no significant growth stimulations were obtained.

The effects of POA and POB when added to 10^{-7} M 2,4-D were also studied (table 7). Both of them apparently antagonize the inhibiting effects of 2,4-D, the effect of POB being conspicuously stronger than that of POA. A more thorough comparison is easily made with the help of tables 8 and 9 which also show the effects of some other substances, especially 1-NMSP. This latter substance has considerably stronger antagonistic effects upon

Table 7. *The effects of POA and POB upon the action of 2,4-D. Values presented as in table 2.*

Exp.	C	n	Root growth, relative values (C = 100)							
			10^{-7} 2,4-D + POA:					POA:		
			0	10^{-6}	10^{-5}	3×10^{-5}	10^{-4}	10^{-6}	10^{-5}	3×10^{-5}
										10^{-4}
26	25.8	4	20.1	20.7	24.4	—	—	99.5	96.5	—
27	26.0	4	19.1	21.9	22.8	—	—	98.9	97.2	—
28	24.0	4	23.0	—	—	30.8	48.4	—	—	91.4
29	26.5	4	18.9	—	—	23.8	40.9	—	—	96.2
			10^{-7} 2,4-D + POB:					POB:		
			0	10^{-6}	10^{-5}	3×10^{-5}	10^{-4}	10^{-6}	10^{-5}	3×10^{-5}
										10^{-4}
30	23.3	4	22.7	25.3	37.5	—	—	103.9	102.9	—
31	24.5	4	25.1	28.7	38.4	—	—	100.4	107.2	—
32	26.1	4	21.4	—	—	71.7	84.0	—	—	99.9
33	25.1	4	23.4	—	—	70.7	82.2	—	—	100.0

Table 8. *Root growth in 10^{-7} M 2,4-D with additions of various substances. Mean values for the experiments recorded in tables 2—4 and 6—7. Data for 1-NMSP and 2-NMSeA from (3). 2-NMSeA = 2-naphthylmethyl-selenide-acetic acid, $C_{10}H_7 \cdot CH_2 \cdot Se \cdot CH_2 \cdot COOH$.*

<i>M</i>	PA	CHA	PB	(—)-POP	POA	POB	1-NMSP	2-NMSeA
	<i>G'</i> = Root growth, rel. values (growth in 10^{-7} 2,4-D = 100)							
10^{-6}	88	96	100	—	109	113	140	263
10^{-5}	59	—	103	100	120	159	301	249
2×10^{-5}	—	—	—	—	—	—	379	171
3×10^{-5}	—	—	—	—	130	319	—	—
4×10^{-5}	—	—	—	—	—	—	351	—
10^{-4}	—	—	—	—	213	372	—	—

Table 9. *Activity coefficients (*A*) for 10^{-7} M 2,4-D in the presence of various antagonists. For the methods of calculation see (3). For POA and POB corrected *G*-values (=22.6 *G'*/100, where 22.6 is the average *G*-value for 10^{-7} M 2,4-D) were used.*

<i>M</i>	POA	POB	1-NMSP	2-NMSeA
10^{-6}	0.88	0.84	0.65	0.38
10^{-5}	0.77	0.55	0.30 (0.31*)	— (0.11*)
2×10^{-6}	—	—	0.18 (0.14*)	— (0.07*)
3×10^{-5}	0.70 (0.66*)	0.28 (0.28*)	—	—
4×10^{-5}	—	—	— (0.09*)	—
10^{-4}	0.40 (0.37*)	0.18 (0.11*)	—	—

2,4-D than has POB, which phenomenon we hypothetically assume to be at least partially connected with the higher affinity of the naphthalene nucleus than of the benzene nucleus to the active positions within the plasm. The difference between POB and POA, on the other hand, emphasizes the role of the side chain for the antiauxin effect, one possible explanation being that a very weak auxin activity remains in the POA molecule, while it is wholly absent from POB.

Discussion

If we try to state explicitly the minimum assumptions which must be made in order to obtain a coherent picture of the available data on auxin and antiauxin effects upon root growth, theses of the following type may eventually be obtained.

- 1) The auxin (*X*) exerts its action when loosely combined with some specific portion of the cytoplasm (*P*).
- 2) The amount of the active complex (*PX*) in the normal root is supra-

optimal for the longitudinal growth, and further applications of auxin causes inhibitions of increasing strength. Due to adaptation phenomena the relation between $[PX]$ and rate of longitudinal root growth is not fix, however, but may change with the pretreatment of the roots, the length of the test period, and so on. The inhibition of the longitudinal growth is probably not caused by any slowing down of metabolic processes (5) but rather by a directional influence upon the growth processes, high PX values leading to the well-known swellings of the root tips.

- 3) An antiauxin (T) is a substance with a conspicuous affinity for P , but which gives a physiologically inactive or very weakly active complex PT . In the root T will compete with the native auxin for the limited amount of P available, the amount of PX will decrease and consequently the longitudinal growth will increase.
- 4) When another auxin (X') is added to X the two auxins will compete for P , but the total amount of active complex ($PX + PX'$) is augmented and the longitudinal growth is decreased.
- 5) The low effect of a weak auxin like PA could conceivably be due either to a low affinity for P , resulting in a low amount of the active complex, or to a low activity of this complex. The very strong effect of an active auxin antagonist (1-NMSP) upon PA (table 5) as well as the absence of antiauxin activity (table 8) speaks in favour of the first assumption, which can be further specified by assigning this low affinity, partly at least, to the presence of a phenyl nucleus instead of e.g. an indolyl, a naphthyl, or a 2,4-dichlorophenyl nucleus.
- 6) For PB, on the other hand, which is slightly less active than PA in repressing root growth, though more difficult to replace by 1-NMSP, it seems necessary to assume a somewhat higher affinity for P , combined with a low activity of the complex P -PB. The increase in affinity as well as the decrease in activity as compared to PA could be caused by the lengthening of the side chain. When PB is added to 2,4-D the total amount of active complex (P -2,4-D + P -PB) is augmented, but this increase is compensated by the lowered average activity of the complex, and the root growth remains almost unaffected.
- 7) The relatively low effect of a weak antiauxin like POB can also be referred to a low affinity for P , which is partly due to the presence of a phenyl nucleus. The still lower effect of POA could possibly be connected with a weak, residual activity of the complex P -POA. Another explanation arises from the possible influences of the type of the side chain upon the affinity for P . As an example of such influences it may be mentioned that substances with »active» nuclei but without a carboxyl group in the side chain (1-naphthyl-acetamide, 2,4-dichloroanisole) do show

only very weak antiauxin activity when tested with the present methods (unpublished results).

The scheme thus outlined is admittedly hypothetical, and omits the complications which may arise from the possible existence of different steps in the growth process, from the differences in dissociation degree of the acids, and so on. It seems, however, that it summarizes a good deal of the available data, and that it may perhaps be of some value for a continued quantitative approach to the problems involved in the differential effects of growth substances upon root growth. Some traits of the scheme may be refound in the diagram of Skoog et al. (25), but there are also divergences. As the growth substances have been characterized by two parameters, neither of which is thought to be exclusively localized to a certain part of the molecule, a purely symbolical representation seems to be preferable at present. If the *affinity* of the substance to *P* is indicated by »Aff», the *activity* of the substance when bound to *P* by »Act», and the intensities of these properties by exponential numbers, symbols of the following type are obtained:

2,4-D: (Aff¹⁰⁰, Act¹⁰⁰). PA: (Aff², Act¹⁰⁰). PB: (Aff⁴, Act³⁰).
1-NMSP: (Aff⁴, Act⁰). 1-NMSeA: (Aff⁴⁰, Act⁰). POB: (Aff¹, Act⁰).

The numbers are only tentative, but it is hoped that it shall be possible to arrive at quantitative estimates by calculation methods based on the Langmuir adsorption formula, which have been devised by Prof. N. Hellström, and found to be well adapted to the material published in (3) (personal communication to the author).

As to the characterization of *phenylacetic acid* (PA) as a weak auxin there seems to be little doubt. It has a very slight or no activity in the *Avena* curvature test (14, 13), the reaction being confined to the tip portion. It is slightly active in the pea test as well as in straight growth of pea stem or *Avena* coleoptile sections (27, 32, 26, 21), is slightly active also in bud inhibition when applied near the bud (31, 13), causes parthenocarpic fruit development and gall formation (13), induces adventitious roots in some species at least (36) but not in etiolated pea stem cuttings (30), and it accelerates the starch hydrolysis in bean leaves (18 a).

For γ -*phenylbutyric acid* (PB) the opinions are more divergent. Thimann & Schneider (27) found a very slight activity (about one fifth of that of PA) in the pea test, in straight growth of *Pisum* and *Avena* sections, and in the *Avena* curvature test. Positive effects on the straight growth of *Avena* sections are also reported by Muir et al. (21), and Went (33) found a slight activity in the pea test when the split pea stems were not washed before the use. In bud inhibition PB is slightly active when applied near the bud (31,

24). All effects are apparently very weak, and interactions with the native auxin might be of importance in causing them. Thus, Went (33) assumes the activity of PB in the pea test to be restricted to a »preparatory reaction» which gives no external manifestations, but which, even in the presence of very minute amounts of a true auxin, may be followed by a conspicuous curvature. In the formation of adventitious roots, in bud inhibition, and in decreasing the coleoptile growth of decapitated but otherwise intact *Avena* seedlings, the effect of PB is thought to be connected with a redistribution of other growth factors (30, 31, 32). Skoog et al. (25) do not accept Went's »hemi-auxin» hypothesis, but classify PB as a weak auxin able to counteract the effects of IA at low concentrations and to exert a »sparing action» on IA at higher concentrations. From the present data no evidence in favour of the hemiauxin nature of PB can be deduced. Its effects in root growth do not seem to differ fundamentally from those of PA, and both may be classified as weak auxins, though of somewhat differing types.

For *cyclohexyl-acetic acid* (CHA) the situation is much the same as for PB, its effect in bud inhibition being comparable to that of PA (31), in straight growth of pea stem sections very slight but positive (32), in the pea test with unwashed material slight and irregular (33). From the present experiments it appears that in root growth the effects of CHA are of principally the same type as those of PA and PB, all of these substances being weak auxins. The absence of a double bond in the ring system of CHA does weaken, but not wholly eliminate the activity. The cause of the slightly deviating inclination of the action curve of this substance (like that of IA) must remain the object of further studies.

The previous data on the effects of *phenoxy-acetic* (POA), *α -phenoxy-propionic* (POP), and *α -phenoxy-isobutyric acid* (POB) have already been summarized (p. 311). The different action of (+)- and (—)-POP parallels that of earlier known pairs of enantiomorphic growth substances (2, 29), the absolute configuration of the active (+)-form being related to that of the active form of the other pairs (11, 12). The change in configuration is most effective in *α -(2-naphthoxy)-propionic acid* (2-NOP), the (+)-form being about 3,000 times more active in repressing root growth than the (—)-form, which exerts a conspicuous anti-auxin effect (2). In POP the difference in activity is only about 100 times, and no antiauxin activity of the (—)-form could be detected. In *α -(1-naphthoxy)-propionic acid* (1-NOP), finally, the (—)-form is most active, but the inhibiting activity is only a few times higher than that of the (+)-form. Apparently the configuration effect is intimately linked to the position of the side chain within the molecule. The effect of the presence of an asymmetric carbon atom in the side chain is also dependent on this position, (+)-POP being 350 times more active than POA,

while (+)-2-NOP is only 4 times more active than 2-NOA, and (—)-1-NOP 7 times more active than 1-NOA. Thus, it seems that the auxin activity is connected with some highly specific steric relations within the molecule, which cannot be strictly described at present, the most promising line of approach being perhaps that tried by Veldstra (28, 29).

Summary

The effects of phenylacetic (PA), γ -phenylbutyric (PB), cyclohexylacetic (CHA), phenoxyacetic (POA), α -phenoxypropionic (POP), (+)- and (—)-form, and α -phenoxyisobutyric (POB) acids on the root growth of young flax seedlings in solution culture (25° C, 6.7 mM phosphate+5 mM Ca-nitrate, pH 5.9) have been determined and compared with the effects of 3-indolylacetic (IA), 2,4-dichlorophenoxyacetic (2,4-D), and α -(1-naphthyl-methylsulfide)-propionic acid (1-NMSP).

PA, PB, CHA and (+)-POP exert an inhibiting effect upon the root growth which is about one hundred times weaker than that of 2,4-D. This inhibition is counteracted by the auxin antagonist 1-NMSP which overcomes the effects of much higher concentrations of PA etc. than of 2,4-D. It is concluded that these substances function as weak auxins, and that their weakness is largely dependent upon a low affinity for the active positions within the plasm. PB approaches the anti-auxin group in some respects, however, the bound molecules having probably a considerably weakened activity.

POA and POB function as auxin antagonists, being able to counteract the inhibition caused by 2,4-D. To obtain the same result much higher concentrations must be used, however, of these substances than of 1-NMSP, and this is assumed to depend on a lower affinity for the active positions within the plasm.

(—)-POP is 100 times less effective in repressing root growth than is (+)-POP, but has no appreciable anti-auxin effects.

This paper is the fourth report on the physiological aspects of the use of growth substances in agriculture (previously published: 1, 2, 3). It is a part of a cooperative investigation, carried out at the Agricultural College of Sweden and supported by the Agricultural Research Council.

References

1. Åberg, B.: On auxin antagonists and synergists in root growth. — *Physiol. Plant.* 3: 447. 1950.
2. — On the growth substance activity of enantiomorphous α -(naphthoxy)-propionic acids. — *Arkiv f. Kemi* 3 (59): 549. 1951.

3. — The interaction of some auxin antagonists and 2,4-D in root growth. — *Physiol. Plant.* 4: 627. 1951.
4. Bonner, J. & Koepfli, J. B.: The inhibition of root growth by auxins. — *Amer. Jour. Bot.* 26: 557. 1939.
5. Burström, H.: The influence of heteroauxin on cell growth and root development. — *Ann. Agric. Coll. Sweden* 10: 209. 1942.
6. — Studies on growth and metabolism of roots. IV. Positive and negative auxin effects on cell elongation. — *Physiol. Plant.* 3: 277. 1950.
7. — Do. VI. The relative growth action of different isobutyric acid derivatives. — *Physiol. Plant.* 4: 470. 1951.
8. — Do. VII. The growth action of α -(phenoxy)propionic acids. — *Physiol. Plant.* 4: 641. 1951.
9. Clauss, H.: Kritische Untersuchungen über den Kressewurzelttest von Moewus. — *Zeitschr. Naturforsch.* 7 b: 112. 1952.
10. Fredga, A.: Studies on synthetic growth substances. I. — *Arkiv f. Kemi* 3 (16): 123. 1951.
11. — & Matell, M.: Do. II. The steric relations between the optically active naphthoxy-propionic acids. — *Arkiv f. Kemi* 3 (47): 429. 1951.
12. — & — Do. III. The steric relations of the optically active α -phenoxy-propionic acids. — *Arkiv f. Kemi* 4. 1952.
13. Gustafson, F. G.: A comparative study of different methods of determining activities of growth-promoting substances. — *Amer. Jour. Bot.* 30: 649. 1943.
14. Haagen Smit, A. J. & Went, F. W.: A physiological analysis of the growth substance. — *Proc. K. Akad. Wetensch. Amsterdam* 38: 852. 1935.
15. Hitchcock, A. E. & Zimmerman, P. W.: Structure of phenoxy compounds in relation to root-inducing activity. — *Amer. Jour. Bot.* 29: 11 S. 1942.
16. — & — Methods of rating the root-inducing activity of phenoxy acids and other growth substances. — *Contr. Boyce Thompson Inst.* 14: 21. 1945.
17. Koepfli, J. B., Thimann, K. V. & Went, F. W.: Phytohormones: Structure and physiological activity. I. — *Jour. Biol. Chem.* 122: 763. 1938.
18. Lundegårdh, H.: The growth of roots as influenced by pH and salt content of the medium. A contribution to the theory of growth. — *Ann. Agric. Coll. Sweden* 10: 31. 1942.
- 18a. Mitchell, J. W., Kraus, E. J. & Whitehead, M. R.: Starch hydrolysis in bean leaves following spraying with alpha-naphthaleneacetic acid emulsion. — *Bot. Gaz.* 102: 97. 1940.
19. Moewus, F.: Der Kressewurzelttest, ein neuer quantitativer Wuchsstofftest. — *Biol. Zentralbl.* 68: 118. 1949.
20. —, Moewus, L. & Skwarra, H.: Nachweis von zwei Wuchsstoffen in Samen und Wurzeln der Kresse (*Lepidium sativum*). — *Planta* 40: 254. 1952.
21. Muir, R. M., Hansch, C. H. & Gallup, A. H.: Growth regulation by organic compounds. — *Plant Physiol.* 24: 359. 1949.
22. Osborne, D. J. & Wain, R. L.: Studies on plant growth-regulating substances. II. Synthetic compounds inducing morphogenic responses in the tomato plant. — *Jour. Hort. Sci.* 26: 60. 1950.
23. — & — Do. III. The production of parthenocarpic pomaceous fruits by chemical treatment. — *Jour. Hort. Sci.* 26: 317. 1951.
24. Overbeek, J. van: Auxin distribution in seedlings and its bearing on the problem of bud inhibition. — *Bot. Gaz.* 100: 133. 1938.

25. Skoog, F., Schneider, C. L. & Malan, P.: Interactions of auxins in growth and inhibition. — *Amer. Jour. Bot.* 29: 568. 1942.
26. Steiner, M.: Ein Beitrag zur Konstitutionsspezifität der Heteroauxinwirkung. — *Planta* 36: 131. 1948.
27. Thimann, K. V. & Schneider, C. L.: The relative activities of different auxins. — *Amer. Jour. Bot.* 26: 328. 1939.
28. Veldstra, H.: Researches on plant growth substances. IV—V. Relation between chemical structure and physiological activity. I—II. — *Enzymologia* 11: 97, 137. 1944.
29. — & Westeringh, C. van de: Do. XIX. Do. IV. Partially hydrogenated naphthoic acids and α -alkyl-phenylacetic acids. — *Rec. Trav. Chim. Pays-Bas* 70: 1113. 1951.
30. Went, F. W.: The dual effect of auxin on root formation. — *Amer. Jour. Bot.* 26: 24. 1939.
31. — Some experiments on bud growth. — *Amer. Jour. Bot.* 26: 109. 1939.
32. — A case of correlative growth inhibition in plants. — *Amer. Jour. Bot.* 26: 505. 1939.
33. — Further analysis of the pea test for auxin. — *Bul. Torrey Bot. Club* 66: 391. 1939.
34. Zimmerman, P. W. & Hitchcock, A. E.: The aerosol method of treating plants with growth substances. — *Contr. Boyce Thompson Inst.* 13: 313. 1944.
35. — & — Formative effects of several substituted phenoxy acids applied to *Kalanchoe*. — *Contr. Boyce Thompson Inst.* 15: 421. 1949.
36. — & Wilcoxon, F.: Several chemical growth substances which cause initiation of roots and other responses in plants. — *Contr. Boyce Thompson Inst.* 7: 209. 1935.

Sensitivity to 2,4-D of Barley and Oats at Different Stages of Development

By

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Experiments carried out in several countries have shown that barley is susceptible to growth regulators at an early stage of development and during the period of anthesis. Oats is generally considered to have a period of susceptibility too.

An earlier investigation showed that, in this country, a considerable decrease in yield may be caused by spraying with 2,4-D during the susceptible period (Andersen and Hermansen 1950). An accurate determination of the susceptible stage is therefore important. The aim of the present investigation was to show correlation between stage of development, abnormalities, and reduction in yield. An earlier paper dealt with a method for determining development stages (Andersen 1952). The present paper deals with the connection between stage of development and effect of spraying.

A pronounced frost effect was observed in the experiments and given due consideration.

Literature

Dersheid (1950) sprayed barley at 9 different dates. He concluded that yield reduction was brought about in three different ways: 1) damage to the vegetative primordium by early application, 2) damage to the spikelets during differentiation, and 3) spraying during anthesis. Olson *et al.* (1951) sprayed barley in 3 experiments. They sprayed at intervals of a few days from pre-emergence to after heading. One susceptible stage was found when the plants were 1—5 inches tall, and another one some days before heading. Many spikelet abnormalities and a considerable decrease in yield were found in the early period of susceptibility. Dersheid (1951)

summarized the results of several workers. Barley was found to have one period of susceptibility before the plants had 3 leaves and another one from the boot stage to the fully headed stage. Oats showed the highest susceptibility between the 4 leaf stage and the boot stage.

In Denmark research work with growth regulators was started at the Agricultural College in 1947. 7 varieties of barley and 7 varieties of oats were sprayed at 4 different times and the morphology of the abnormalities was studied in detail (Pedersen *et al.* 1948). Based on the results some types of abnormalities were defined and counted in experiments 1948 (Andersen and Hermansen 1950). Most abnormalities were caused by spraying with 1 kg 2,4-D per ha, when the number of leaves was 3.0 in barley and 4.6 in oats. The length of spike and panicle was 0.48 and 0.86 mm respectively. The dates for greatest number of abnormalities and lowest yield were identical. The results for barley agree with results found in England (Large and Dillon Weston 1952). Those investigators worked mainly with ear distortions in barley. They determined the stage of development by leaf count.

A survey of further literature on the effects of different times of spraying is given by Åberg (1949), Olson (1949), and Olson *et al.* (1951).

Varietal differences are reported, but Åberg (1949), who reviewed some experiments, did not find that varietal differences were demonstrated with certainty, since the stage of development of the varieties may have been different. Olson (1949) reported some experiments, but did not always find agreement between the results. A considerable work was done by Middleton *et al.* (1948), but only one spraying was used.

Some occasional notes are given about the effect of frost. Eggink (1950) found in experiments in 1949 a low yield after spraying on May 10. He assumed that the low yield was due to frost on the two preceding nights. Jacobsen and Sundelin (1950) also reported an effect of frost. In the investigations of Olson *et al.* (1951) frost occurred during one experiment with barley. The susceptible period was prolonged, but Olson (1952) did not consider this an effect of the frost.

Experimental Procedure

Most of the experiments were located at Albertslund near Copenhagen, but in 1951 one was placed at a farm near Aarhus.

1949. One variety of barley (Rigel) and one variety of oats (Stål) were sprayed at several dates, applying each time 1 kg sodium salt of 2,4-D per ha. The size of the plots was 1.2 by 1.2 m and no yield determination was made.

1950. 4 varieties of barley were included: Freja, Alfa, Maja, and Rigel; and 4 varieties of oats: Minor, Rex, Opus, and Stål. The commercial preparation Herbatox D was applied at a rate corresponding to 1 kg sodium salt of 2,4-D per ha at 7 different dates. The size of the plots was 2.55 by 2.25 m, and there were 5 replications. In oats a small experiment was sprayed with 4 kg per ha in order to secure a fairly large number of abnormalities. The abnormalities in oats were counted in samples from these plots. The yield was determined only in plots sprayed with 1 kg per ha.

1951. The plan was the same as in 1950. The experiment was repeated with barley and oats at Albertslund and an experiment with barley was placed near Aarhus.

All experiments were carried out on clean soil or kept weed-free by hand-hoeing. At Albertslund the soil is deficient in manganese. Therefore in 1950 and 1951 the crop was sprayed with manganese sulfate after spraying with 2,4-D.

Abnormalities were counted by the same method as was used earlier (Andersen and Hermansen 1950). However, for simplification the term »abnormal kernels» is applied. This term comprises 3 kernels from the abnormality called »whorl», both kernels from »supernumerary spikelets», and one kernel from »opposite spikelets» and other abnormalities.

The method used for determination of stage of development, and a scale of stage of spikes and panicles, are described in another paper (Andersen 1952).

Effects on Barley

The stage of development of the spike was nearly the same in all four varieties. The spike started growth with a lower number of leaves in the variety Freja than in the variety Alfa (Andersen 1952), but the difference was modified, because Alfa had a higher number of leaves than the other varieties. Average figures may therefore be used for stage of development, abnormalities, and yield. The greatest number of abnormalities in all varieties was found after spraying at the same date, and a statistical treatment of the figures for yield showed no interaction between variety and time.

The highest number of »tubular leaves» followed treatments applied when the stage of the spike was between 1.0 and 1.5 (Figure 1). Few followed treatments applied before the spike had reached stage 1 and few after stage 2. In the present experiments plants were not sprayed until stage 1, but in some other experiments the first spraying took place at the pre-emergence stage. In the latter experiments few abnormalities were found before the plants reached stage 1.

The highest number of abnormal kernels followed treatments applied when the stage of the spike was between 1.5 and 2.5. Barley seems to produce most spikelet abnormalities when the stage of the spike is about 2.0. A high number of abnormal kernels was found at later stages in 1949 and 1951. This can be explained as an effect of frost and will be discussed below.

Stage 2 is reached when the shoot apex begins to elongate. The change from the vegetative to the reproductive phase occurs possibly at stage 2, but is not visible before stage 4 is reached. Stage 4 is characterized by visible spikelet primordia, and was usually reached 6 days later than stage 2.

Preliminary investigations in 1951 showed that the first ear distortions could be seen in dissected plants 4 to 5 days after spraying. After 6 to 7 days the increase in the number of abnormal ears was only small. This observation seems to indicate that the abnormal kernels are caused by distortions in cell divisions when the change from the vegetative to the reproductive

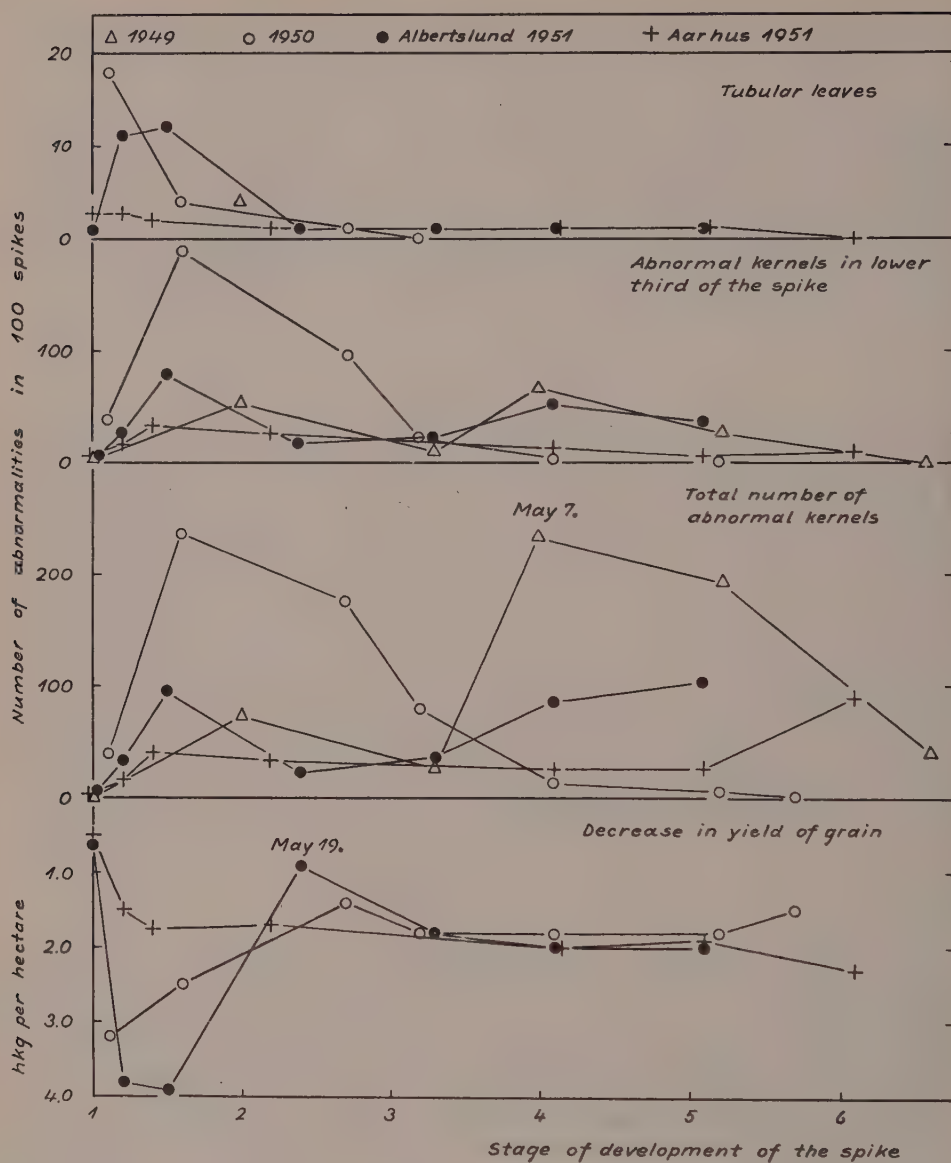


Figure 1. Effects on barley from 2,4-D treatments at different stages of development. Figures from one variety (Rigel) grown in 1949 and average figures from 4 varieties grown in 1950 and 1951. 2,4-D applied at a rate of 1 kg sodium salt per ha.

phase takes place. »Tubular leaves» are distortions of the growth of the last leaf, and it is not surprising that this abnormality was found before the change to the reproductive phase occurred.

At Albertslund in 1951 the number of abnormal kernels was rather small on May 19 when the stage of the spike was 2.4. However, another experiment showed a very low effect of spraying about May 20 (Figure 2).

There is a rather large difference in the number of abnormalities found at the early stage in the different experiments. This cannot be fully explained, but it should be noted that temperature was considerably higher in 1950 than in 1949 and 1951.

At an early stage of development most abnormal kernels, 70 to 80 per cent, were in the lower third of the spike. At later stages the proportion of abnormal kernels in the lower third was rather small, even if a high number was found.

Due to the large variation between the number of abnormal kernels at different stages, the standard error varies from stage to stage. However, in each experiment a standard error was calculated for the date on which spraying caused most abnormal kernels. The calculations show a standard error of 12 abnormal kernels per 100 spikes in 1950 and a standard error of 5 abnormal kernels in the two experiments in 1951.

In the untreated plots the yield of grain was 37.9 hkg per ha at Albertslund in 1950, 48.5 hkg at Albertslund in 1951, and 33.1 hkg at Aarhus in 1951. No significant reduction in yield was found at Aarhus, but here also the number of abnormalities was small. At Albertslund in 1950 and 1951 yield differences of 1.6 and 3.4 hkg respectively may be considered significant at the 1 per cent level. In both years the large yield reduction coincided with many »tubular leaves».

The large yield reduction in plots with »tubular leaves» may be explained as a result of that abnormality. In 1950 and 1951 the average yield of grain from normal spikes was 0.77 gr. per spike. The yield of spikes from »tubular leaves» was 0.40 gr.

Yield reduction caused by abnormal kernels was not definitely proved. However, in 1950 when the greatest number of abnormal kernels was found, the yield was rather low.

The susceptibility depends no doubt on development stage of the spike at spraying time, therefore it was desirable to study the effect as correlated to that stage. However, in practice leaf count may be used instead. Only very few abnormalities were found before all plants had emerged, but when the number of leaves was 1.4 a high number of »tubular leaves» was observed in one case. The greatest number of »tubular leaves» and of abnormal kernels were generally found when the number of leaves was about 2.0, with some variation from year to year (Table 1). The susceptible stage may be con-

Table 1. *Barley. Stage of development when an optimum of abnormal kernels was found.*

	Albertslund 1949	Albertslund 1950	Albertslund 1951	Aarhus 1951
Stage of spike	2.0	1.6	1.5	1.4
Number of leaves	1.7	2.54	2.24	1.97

sidered as ended when the stage of the spike is 3.0. In all experiments that stage was reached before the number of leaves was 3.5. At Albertslund in 1949 and at Aarhus in 1951 stage 3 was reached when the number of leaves was about 2.5 (Andersen 1952).

Influence of Frost

In 1949 many abnormal kernels were caused by treatment when the development stage of the spike was 4.0 and 5.3 (Figure 1). In 1951 a considerable number was found at a late stage of development in both experiments (Figure 1). At those late stages spikelet primordia were formed on the main shoots. It seems unlikely, therefore, that abnormalities should appear in the main shoots, and they are probably formed in the tillers. Olson (1952) has shown that abnormalities appear at a later date in the tillers than in the main shoots. Most abnormal kernels were found in the middle and the upper third of the spike, which is susceptible at a later date than the lower third. At the early susceptible stage most abnormal kernels were found in the lower third (Figure 1).

The upper third of the tillers does not generally show abnormal kernels, and the effect of 2,4-D seems to have been strengthened considerably in plots sprayed shortly before or after a frosty night. In 1949 plants in stage 4.0 and 5.3 were sprayed on May 7 and May 11 respectively. On May 9 there was frost. In 1951 frost or very low temperature was noted at Albertslund 4 days after the last spraying. At Aarhus frost was noted 2 days after the last spraying.

Frost effect appeared in an experiment from 1951, in which barley was sown at 10 different dates. The plots of each sowing were divided into three sections. One of the sections was sprayed when the plants had 2.0 leaves, another when the number of leaves was 2.5, and the third when the plants had 3.0 leaves. Plants from three different sowings having 2.0, 2.5 and 3.0 leaves were sprayed the same day. Variations in number of leaves between the different dates of spraying were not very great. There were slight dif-

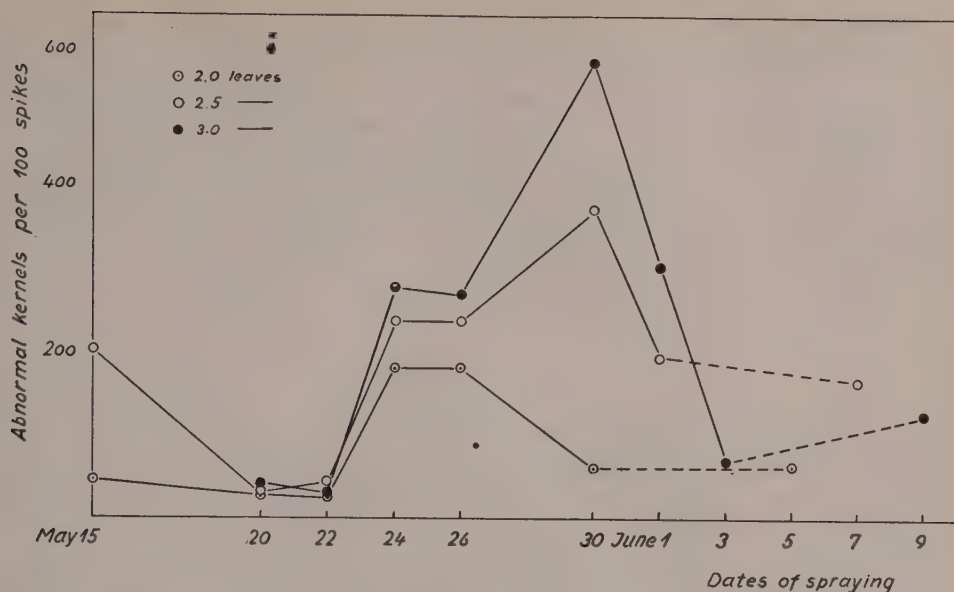


Figure 2. Variations in effect on barley sprayed at the same stage of development. Barley was sown at 10 different dates and the plots of each sowing were divided into three sections. Plants from three different sowings having 2.0, 2.5, and 3.0 leaves were sprayed on the same day. 2,4-D was applied at a rate of 1 kg sodium salt per ha.

ferences in the stage of spike development, but these seem to be of little importance.

The number of abnormal kernels is shown in Figure 2. On May 15 the number was rather high. On May 20 and 22 the number was very low, but after that day the number increased and reached a maximum on May 30. Spraying of the 3-leaf stage at this date gave 5—6 abnormal kernels per spike. After that the number of abnormal kernels decreased. Eighth and ninth sowing failed due to a bad emergence, but the tenth sowing was sprayed on June 5, 7, and 9, and the number of abnormal kernels was not unusually high.

After spraying on May 30, 8.5 per cent of all the spikes were branched. Such high percentages is unusual. In the rest of the experiment only 0.2 per cent was found.

On May 27—29, 33 mm of rain fell. About noon on May 29 the rain stopped, and on May 30 there was bright sunshine in the morning. At the author's house the grass was stiffened by rime at 5 in the morning, but the rime was melted by the sun before 6. At Albertslund no rime was visible at 6, but the temperature was very low.

The experiment has shown a quite different effect when plots are sprayed at the same stage of development. The small number of abnormal kernels after spraying on May 20 and 22 is probably due to weather conditions, but a satisfactory explanation cannot be given at present. The high number of abnormal kernels and of branched spikes after spraying on May 30 may be due to a frost effect. The high number of abnormal kernels found after spraying on May 24, May 26, and June 1 indicate damage if plots are sprayed some days before or some days after frost.

Effects on Oats

Average figures are used for the 4 varieties grown in 1950 and 1951. The development stage of the panicle of Minor was a little more advanced than the stage of the panicle of the other varieties, and the susceptible period seemed to begin and end 2—3 days earlier in Minor than in the other varieties. However, the long period of susceptibility justifies the use of average figures, and no significant interaction was found between variety and time with regard to yield.

Oats is susceptible for a longer period of time than barley, probably because the oat panicle needs more time for its differentiation than the barley spike. Most abnormalities develop during a long period of time and are difficult to count. In Figure 3 only the figures for »branching» and »non-split lemmas» are shown. These abnormalities are comparatively easy to count and are found in great numbers only at a certain stage of development. »Branching» was most common when the stage of panicle was about 3. »Non-split lemmas» were found when the stage of the panicle was between 3 and 6 (Figure 3).

In 1950 and 1951 the dates for the highest number of »non-split lemmas» and lowest yield were identical. In 1951 a large yield reduction followed treatments applied at all stages between 3 and 7, but treatments applied before stage 3 and after stage 7 caused no severe reduction. In 1950 all treatments applied after stage 3 caused a large reduction. That year spraying was not continued late enough to show whether yield reduction would have been less severe by later spraying as was the case in 1951. A yield reduction of 2.4 hkg per ha in 1950 and 2.0 hkg in 1951 was significant at the 1 per cent level.

Stage 3 was reached very early in 1951, in the variety Rex, Opus, and Stål when the number of leaves was about 3.5. In 1950 stage 3 was reached by the same 3 varieties when the number of leaves was 4.0, in 1949 by the variety Stål when the number was 4.5. Stage 7 was usually reached when

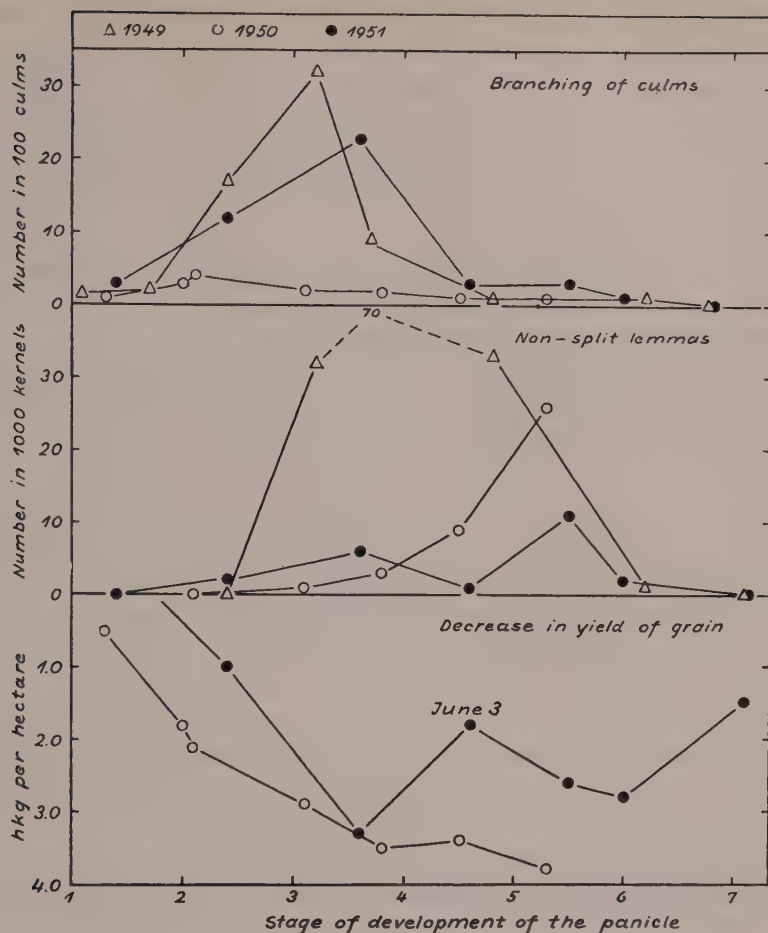


Figure 3. Effects on oats from 2,4-D treatments at different stages of development. Figures from one variety (Stål) grown in 1949, and average figures from 4 varieties grown in 1950 and 1951. Abnormalities from plots sprayed with 4 kg sodium salt of 2,4-D per ha. Yield from plots sprayed with 1 kg per ha.

the plants had 2 leaves more than at stage 3, in 1951 when the number of leaves was 5.5, in 1950 and 1949 when the number of leaves was 6.0 and 6.5. In the variety Minor, not commonly grown, the susceptible period began and ended when the plants had half a leaf less than the other varieties.

The effect of spraying on June 3 in 1951 was very small. Only one »non-split lemma» was found after spraying at that date, but six were found after spraying on May 31 and 11 after spraying on June 5. Also the yield reduction from spraying on June 3 was small. This is probably due to weather condi-

Table 2. *Lodging resistance in oats 1951. Mean of four varieties.*
1 = upright, 10 = completely lodged.

Check	May 21	26	31	June 3	5	7	11
1.5	1.6	1.7	3.8	4.4	4.7	4.5	2.7

tions, but it is not clear whether the effect of the growth regulator was strong on May 31 or weak on June 3.

In 1951 there was a heavy gale a fortnight before harvest, and the oat crop was partly lodged. The lodging was most severe in plots sprayed between May 31 and June 7 (Table 2). The stage of panicle on those dates was 3.6 and 6.0, the number of leaves 3.94 and 5.29. That agrees with the results from 1948, where lodging was most severe when the number of leaves was between 3.9 and 5.9 (Andersen and Hermansen 1950).

Differences between Varieties

Varietal differences in response to 2,4-D are rather difficult to demonstrate. For this purpose the varieties in question must be sprayed at the same stage of development and at the same date.

The present varieties of barley developed nearly identically. Therefore, and because the plants were sprayed from the beginning to the end of the susceptible period, comparison is justified. The oat varieties, also, developed nearly identically. Here no varietal difference was found.

A considerably higher number of abnormalities was found in Maja than in any other barley variety, and there were more in Rigel than in Freja and Alfa (Table 3). No significant difference in yield reduction was observed,

Table 3. *Difference between varieties. Total number of abnormalities in the experiments in 1950 and 1951, and average figures for yield decrease.*

Barley	Freja	Maja	Alfa	Rigel	Diff. 1 % level
Opposite spikelets	1154	1958	1123	1672	448
Whorl	133	446	120	232	297
Abnormal kernels	2457	4836	2752	3602	547
Tubular leaves	152	235	189	149	(61) ¹
Yield reduction hkg/ha	2.2	2.4	1.1	2.0	n. s.
Oats	Minor	Rex	Opus	Stål	
Yield reduction hkg/ha	3.4	2.4	1.1	1.7	n. s.

¹ At 5 % level only.

but yield reduction of Alfa was in all experiments less than that of Maja and Freja.

No significant difference in yield reduction was found in oats, but Minor seems to be most susceptible to spraying damage. The same result was found earlier (Pedersen *et al.* 1948) and there are reasons to stress the importance of care in spraying that variety.

Discussion

The development stage is determined by the method described by Andersen (1952). Barley was found to be most susceptible before stage 3 was reached. That agrees with 1948 results (Andersen and Hermansen 1950), although small differences are found. 1 kg 2,4-D caused most damage in 1948 on May 4 when the spike was 0.48 mm long. No analysis of the stage of development of the spike was made, but the stage can be estimated to have been about 2.5. The estimate is based on photos, on measurement of the length of the spike and on the relative number of abnormal kernels found in the lower third of the spike. The most susceptible period was probably passed in the preceding week, in which there was no spraying. From the results found in 1950 and 1951 no decrease in yield at that late stage was to be expected. No explanation for this can be given, but it should be pointed out that the number of abnormalities was very high in 1948.

There was a large decrease in yield of oats as a result of spraying with 1 kg 2,4-D in 1948 when the number of leaves was 4.6. The stage of panicle can be estimated to have been about 4 at spraying time. No serious yield reduction was found when the stage was less than 3 or more than 7. That agrees with results from 1950 and 1951.

In many cases an analysis of the ear is not possible. In such cases only the less reliable statement of the number of leaves can be made. No serious damage was found when the barley spike had reached stage 3 at spraying time. In all experiments from 1948—1951 this stage was passed when the number of leaves was 3.5. In some years the spike had reached stage 3 already when the number of leaves was 2.5. In other years barley was highly susceptible when the plants had 2.5 leaves. The highest susceptibility was generally found when the plants had 1.5 to 2.0 leaves.

The susceptible period in oats is rather long, and good weed control is obtainable by spraying during that period. The risk of damage by spraying is therefore higher in oats than in barley. Serious spraying damage has been found on stages between 3.5 and 6.0 leaves, but the limits of the susceptible

period is not the same in all years or in all varieties. In all varieties and in all years damage was found when the number of leaves was about 5.

The results agree with results obtained in Canada and U.S.A. (Olson *et al.* 1951, Dersheid 1951). Those investigators showed that barley had a period of susceptibility before the plants reached the 3 leaf stage. Oats was susceptible at a later stage but before the boot stage. They also found that barley has another period of susceptibility shortly before heading.

Weather conditions seem to be rather important, especially frost. Having passed the period of susceptibility barley still is highly affected if frost occurs. The effect was noticed both in plots sprayed before the day of frost and in plots sprayed after.

Summary

1. Four varieties of barley were sprayed with 2,4-D in 3 experiments, and 4 varieties of oats were sprayed in 2 experiments. In each experiment sodium salt of 2,4-D was applied at 7 stages of development. The rate of application was 1 kg per ha. In a small experiment with one variety of barley and oats 2,4-D was applied at different dates.

2. Barley is most susceptible about 6 days before the spikelet primordia can be seen under the microscope. When the ear reaches stage 3 (see Andersen 1952) the most susceptible period is passed. That stage of development was reached in all experiments before the plants had 3.5 leaves. The dates for the greatest number of the abnormality »tubular leaves» and the lowest yield were identical.

3. Oats is most susceptible when the stage of development of the panicle is between 3 and 7. This corresponds nearly to a number of leaves between 4 and 6. The dates for greatest number of the abnormality »non-split lemmas» and the lowest yield were identical.

4. Many abnormal kernels were found in 1951 at a late stage of development. This can be explained if we suppose that frost strengthens the effect of 2,4-D. Weather conditions seem to have been of considerable importance in 1951.

5. Varietal differences with regard to abnormalities were found in barley.

The author is indebted to Prof. Axel Pedersen and Mr. J. E. Hermansen for help and discussions concerning this and the previous work. The works were aided by a grant from The Danish Agricultural Cereal Breeding Foundation.

References

- Åberg, E.: Hormonderivat i kampen mot ogräs VIII. Översikt över resultaten från åren 1946 till 1948. — *Växtodling* 4: 73. 1949. (Engl. summary).
- Andersen, S. & Hermansen, J. E.: Effect of hormone derivatives on cultivated plants II. Spraying of cereals with 2,4-D and 4K-2M at different dates. — *Kgl. Vet.- og Landbohøjskoles Årsskrift*, København 1950: 141.
- Andersen, S.: Methods for determining stage of development in barley and oats. — *Physiol. Plant.* 5: 199. 1952.
- Dersheid, L. A.: Physiological responses of barley to 2,4-D. — *Proc. Seventh Ann. North Central Weed Control Conf.* 1950: 99.
- Control of weeds in field crops. Spring sown grain. — *Res. Rpt. Eighth Ann. North Central Weed Control Conf.* 1951: 53.
- Eggink, H. J.: Onkruidbestrijding in granen. — *Versl. Centr. Inst. landbouwk. Onderzoek.* 1949: 119.
- Jacobsen, G. & Sundelin, G.: Kemisk ogräsbekämpning. — *Statens Jordbruksförsök. Särtryck* 45. 1950.
- Large, E. C. & Dillon Weston, W. A. R.: Ear distortion in barley and other cereals caused by spraying with MCPA and 2,4-D. — *J. Agric. Sci.* 41: 338. 1952.
- Middleton, G. K., Hebert, T. T., & Klingman, G. C.: How do oat varieties react to 2,4-D. — *Crops and Soils.* 1948.
- Olson, P. J.: Effects of 2,4-D on spring planted small grains. — *Proc. Sixth Ann. North Central Weed Control Conf.* 1949: 67.
- Personal communication. 1952.
- Olson, P. J., Zalik, S., Breakey, W. J. & Brown, D. A.: Sensitivity of wheat and barley at different stages of growth to treatment with 2,4-D. — *Agron. Jour.* 43: 77. 1951.
- Pedersen, A., Andersen, S. & Hermansen, J. E.: Hormonderivaternes virkning på kulturplanterne I. Sprøjtning af byg og havre med 2,4-D og 4K-2M. — *Kgl. Vet.- og Landbohøjskoles Årsskrift*, København 1948: 101. (Engl. summary).

On Detrimental Effects of High Light Intensities on the Photosynthetic Mechanism

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(Received March 7, 1952)

1. Introduction

In previous publications (Steemann Nielsen 1942, 1949) a temporary inhibition of the rate of photosynthesis was shown in certain cases after changing from high to low light intensity. In exceedingly bright light a decrease in the rate of photosynthesis was further shown without changing to a lower light intensity, an effect described also by Stålfelt (1939) and Myers and Burr (1940).

Whereas the last-mentioned effect according to all experiences is due to a chlorophyll-sensitized photo-oxidation of one or more of the enzymes participating in photosynthesis, the after-effect of high light intensity on the rate of photosynthesis at low light intensity is more difficult to explain. Here, however, no photo-oxidation of an enzyme participating in an ordinary chemical reaction during photosynthesis can be responsible. The chemical (non-photochemical) reactions limit the rate of photosynthesis only at high light intensities. At low light intensities the limitation is taken over by the photochemical reactions. A destruction of an enzyme participating in a chemical reaction, therefore, must first of all assert itself at high light intensities.

According to these considerations the temporary inhibition of the rate of photosynthesis after changing from high to low light intensity was supposed to be a result of changes in the photochemical part of the photosynthetic mechanism. The phenomenon was called reversible inactivation of chlorophyll (Steemann Nielsen 1949). Although there is yet no direct indication that

chlorophyll as such is affected in bright light, the plants act as if a considerable part of the chlorophyll is in an inactive state after the light has been changed from a high to a low intensity. Inactivation of chlorophyll or of another part of the photochemical mechanism must assert itself only at low light intensities, where the rate of the photochemical reactions is the limiting factor for photosynthesis. At a sufficiently high light intensity a partial inactivation of e.g. chlorophyll will produce no reduction at all of the rate of photosynthesis. No matter how the photochemical mechanism in photosynthesis is blocked in bright light, the result will be just like inactivation of chlorophyll. This designation, therefore, must be considered rather characteristic.

No real discussion of the question how the photochemical mechanism in photosynthesis may be blocked in bright light will be given here. The experiments published in this article do not seem to give any decisive indications in this direction.

It is a question whether the above mentioned inactivation is a link in photosynthesis itself or not. If it is, the rate of reactivation of the photochemical system must normally be so high that even in bright light no real blocking of the photochemical system is brought about. Reactivation, as shown in my papers already cited, is a chemical process, probably involving one or more enzymes. In plant material showing a typical temporary inhibition of the rate of photosynthesis after changing from high to low light intensity the rate of reactivation must be reduced, if inactivation is a normal link in photosynthesis.

If, on the other hand, this is not the case, it seems likely that inactivation is caused by a photo-oxidative decomposition of something of importance for the photochemical reactions in photosynthesis.

In my former publications I was most inclined to suppose inactivation and reactivation to be normal links in photosynthesis. If this is the case, however, a chlorophyll molecule must most likely be inactivated when it has taken part in the assimilation of a definite number of CO_2 molecules. Some experiments with *Fucus serratus* (cf. Steemann Nielsen 1949, p. 261) showed that about 40 CO_2 molecules were assimilated for one molecule of chlorophyll inactivated. If the first free assimilation product had a carbon-chain of about 40 the background for the theory would be established. The recent investigations of Calvin *et al.* (e.g. Benson, Bassham, Calvin, Goodale, Haas and Stepka, 1950) have, however, shown the first free product in photosynthesis to be a simple compound (2-phosphoglycerate) without a long carbon-chain.

2. Is Inactivation Due to a Photo-oxidative Decomposition?

As already mentioned very bright light may produce a chlorophyllsensitized photo-oxidative decomposition of one or more enzymes participating in chemical reactions during photosynthesis. If enzymes thus can be affected, other compounds may be affected, too. The experiments in extraordinarily bright light published in my 1949 paper (see figs. 13 and 14) did not give evidence of destruction of compounds other than enzymes. When changing from a very high light intensity (60,000—160,000 lux), producing a marked decrease in photosynthesis, to a lower, but still high light intensity (21,000 lux), no or only a very insignificant further decrease in the rate of photosynthesis appeared. This is evidence of the fact that the chemical reactions still limit the rate of photosynthesis. If the photochemical part of the photosynthetic mechanism has been affected by the very bright light, the damage has not become so significant that the limitation at 21,000 lux is taken over by the photochemical reactions.

In order to investigate a possible damage in very bright light of the photochemical part of the photosynthetic mechanism simultaneous to the photo-oxidation of the enzymes, some new experiments were made changing the light intensity after an hour at 100,000 lux to the low intensity of 3,000 lux where the photochemical reactions limit the rate of photosynthesis. Figure 1 illustrates a series of these experiments. *Cladophora insignis*, the same species as was employed in my 1949 article, was used. The methodics for measuring the rate of photosynthesis were the same, too (O_2 -production in vigorously stirred water by means of a Winkler-titration). HCO_3^- -ions were in excess ($3 \cdot 10^{-3}$ equiv./l.).

In order to increase the precision of the determination of photosynthesis the oxygen tension at the start of all individual experiments was kept at exactly the same level. Experiments have shown that variations in the initial oxygen tension may have some — although not very significant — influence on the determination of the apparent rate of photosynthesis. The vessel into which the plants were dipped for washing before starting a new experiment, was filled with the same kind of water as used in the experiments.

The rate of photosynthesis (Figure 1) decreased about 40 per cent. during the first hour at 100,000 lux. After changing to 3,000 lux the rate of photosynthesis decreased to such an extent that respiration (80 relatively) was larger than photosynthesis. The rate of total photosynthesis (real assimilation) in the first 10 minute-experiment was only 17 per cent. of the normal rate at 3,000 lux (measured before the experiments at 100,000 lux). After 80 minutes at 3,000 lux photosynthesis had only increased to 45 per cent. of the normal rate. The *Cladophora* then was placed by a window facing

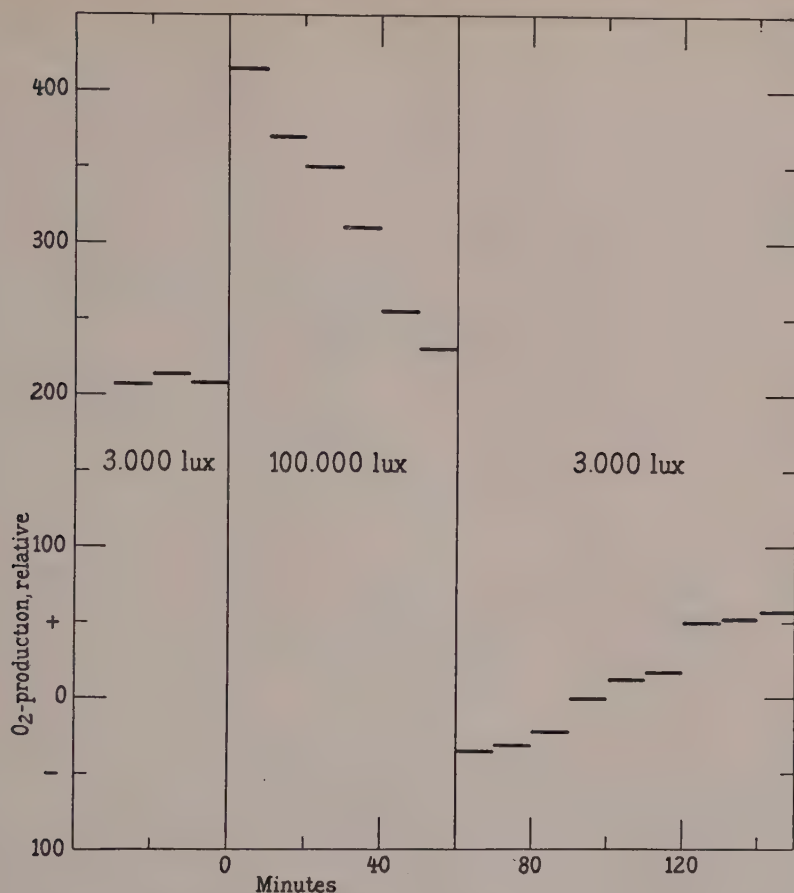


Fig. 1. Photosynthesis in *Cladophora insignis*. 18° C., pH 8. 23.8.1949.

north. The rate of photosynthesis at 3,000 lux was measured the next day and found quite normal. The reestablishment of the rate of photosynthesis at 3,000 lux after pretreatment for 60 minutes at 100,000 lux was slow in the material of *Cladophora* in question. In an identical series of experiments made two days later only 62 per cent of the normal rate of photosynthesis was found after 5 hours at 3,000 lux following 60 minutes at 100,000 lux.

Light is not necessary for the reestablishment of photosynthesis at low light intensity after pretreatment at 100,000 lux. Figure 2 shows a series of experiments. After 60 minutes at 100,000 lux the *Cladophora* (the same material as used in the experiments shown in fig. 1) was placed in the dark

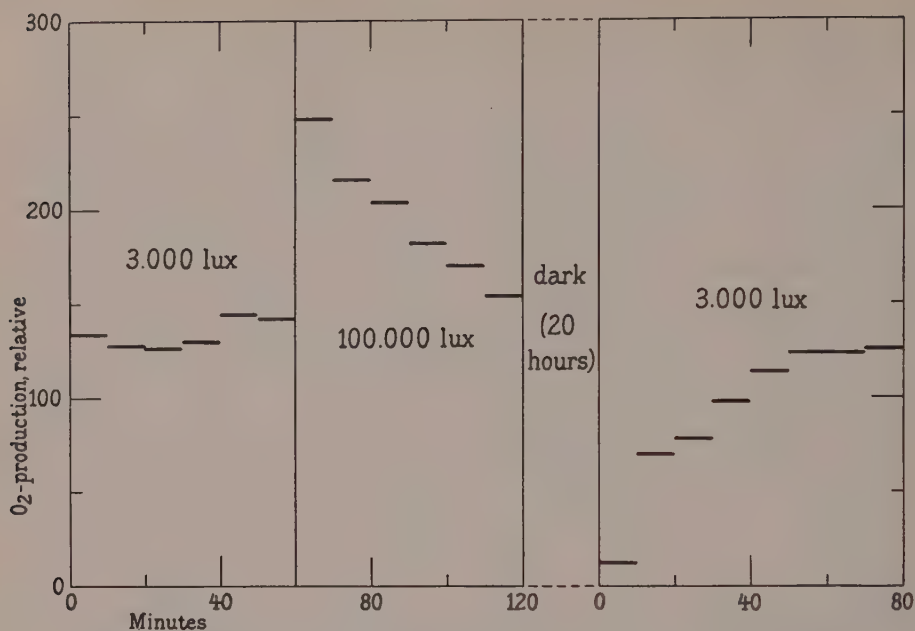


Fig. 2. Photosynthesis in *Cladophora insignis*. 18° C., pH 8. 26.8.1949.

for 20 hours. Illuminating thereupon with 3,000 lux it took only 50 minutes to establish the normal rate of photosynthesis at that light intensity.

The very long time necessary for reestablishing the rate of photosynthesis at low light intensity after the pretreatment at the exceedingly high light intensity indicates that the inactivation of the photochemical system under these conditions is no regular link in photosynthesis. It is to be supposed that the chlorophyll-sensitized photo-oxidation in exceedingly bright light besides attacking one or more enzymes also attacks a link in the photochemical system of photosynthesis.

In order to investigate this possibility further some other experiments were made. Figure 3 shows the dependence of the inhibition of the rate of photosynthesis at 3,000 lux on the time in which the plant has been pretreated with 100,000 lux. After 10 minutes at 100,000 lux and then changing to 3,000 lux a rate of total photosynthesis of 55 per cent of the normal rate was found in the first 10 minutes. The normal rate was obtained after about one hour at 3,000 lux.

If the duration of the pretreatment at 100,000 lux was 60 minutes, the rate of total photosynthesis after changing to 3,000 lux at the start was only 16 per cent of the normal rate. After an hour at 3,000 lux the rate was only

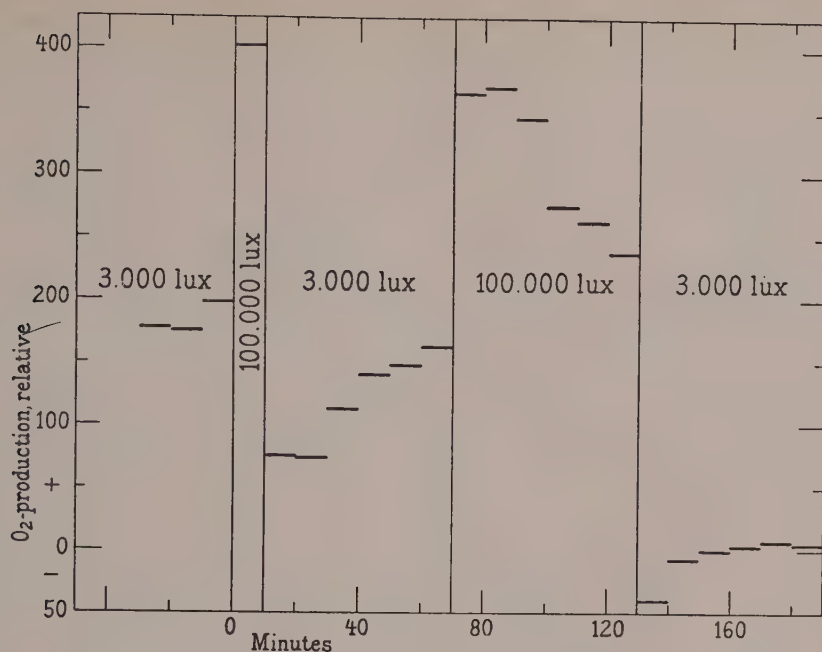


Fig. 3. Photosynthesis in *Cladophora insignis*. 18° C., pH 8. 25.8.1949.

34 per cent of the normal. These experiments clearly support the idea of a photo-oxidative damage of the photochemical mechanism in extraordinarily bright light, since such a pronounced dependence on the time at which the plant is exposed to this light intensity otherwise is difficult to explain.

In order to investigate whether the inactivation of the photochemical system is of the same kind at moderately high light intensities, where it is impossible with certainty to demonstrate photo-oxidation of enzymes participating in the chemical reactions during photosynthesis, the experiments shown in Figure 4 were made. These experiments were made with the same plant material as used in the experiments described in Figure 3, but 2 days before. After 60 minutes preillumination at 17,000 lux the curve showing the reestablishment of the photosynthetic rate at 3,000 lux is nearly identical with that obtained after pretreatment for 10 minutes at 100,000 lux. The duration of the time for establishing a normal rate of photosynthesis was about 50 minutes after 60 minutes at 17,000 lux, and about 70 minutes after 10 minutes at 100,000 lux. As the products of light intensity and time are identical in the two series of experiments during preillumination (1,002,000 lux-minutes, respectively 1,000,000 lux-minutes),

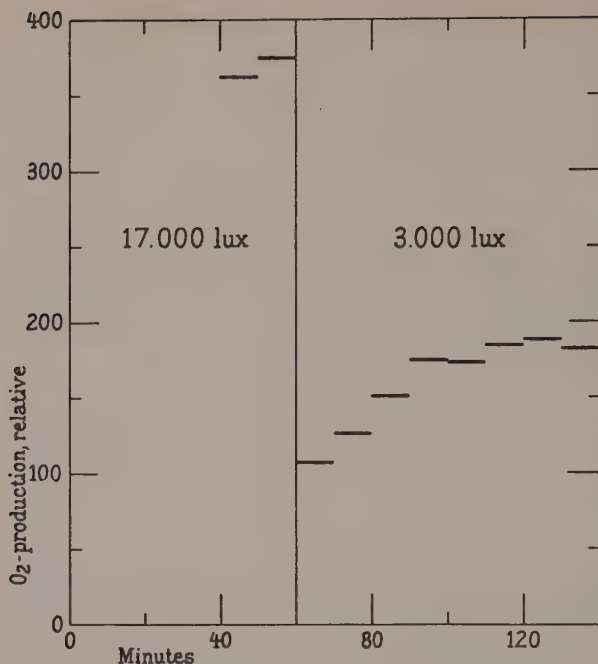


Fig. 4. *Photosynthesis in Cladophora insignis*. 18° C., pH 8. 23.8.1949.

this strongly supports the idea of the inactivation always being due to photo-oxidation. The somewhat longer time required for establishing a normal rate after 100,000 lux is to be expected, as the time for reactivation during the preillumination is 6 times longer at 17,000 than at 100,000 lux. The exactness of the determinations of the duration of the period for establishing the normal rate at 3,000 lux is, however, only slight.

An equilibrium between inactivation and reactivation is further established after a definite time at a high light intensity. According to the *Fucus* experiments described in Figure 26 (Steemann Nielsen 1942) this equilibrium was already established after about 30 minutes at 23,000 lux. In the experiments using extraordinarily high light intensities the equilibrium is possibly not established until photosynthesis has fully ceased. No experiments of sufficient duration have, however, been made.

As shown above photo-oxidation at extraordinarily high light intensities besides attacking enzymes participating in the chemical reactions during photosynthesis also damages a part of the photochemical mechanism. When changing from the extraordinarily high light intensity producing a marked decrease in photosynthesis to a lower, but still high light intensity normally

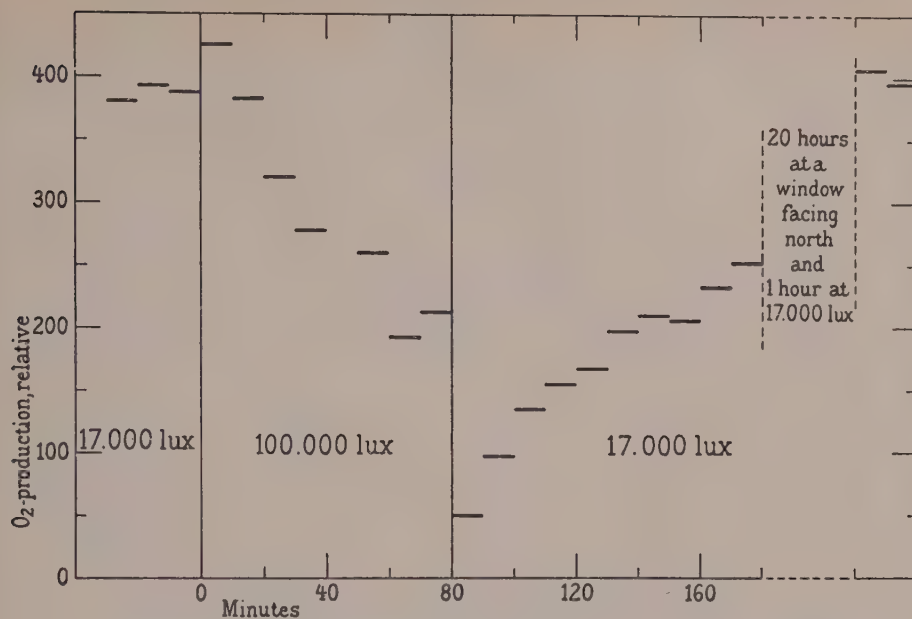


Fig. 5. Photosynthesis in *Cladophora insignis*. 18° C., pH 8. 20.8.1949.

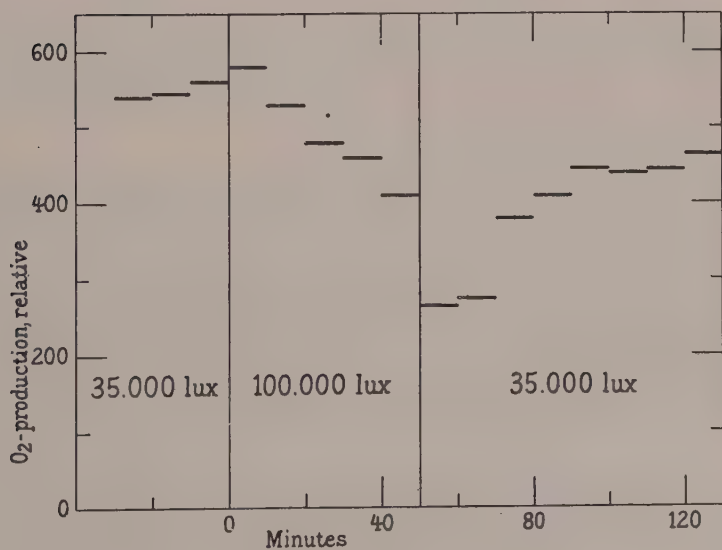


Fig. 6. Photosynthesis in *Cladophora insignis*. 18° C., pH 8. 13.9.1949.

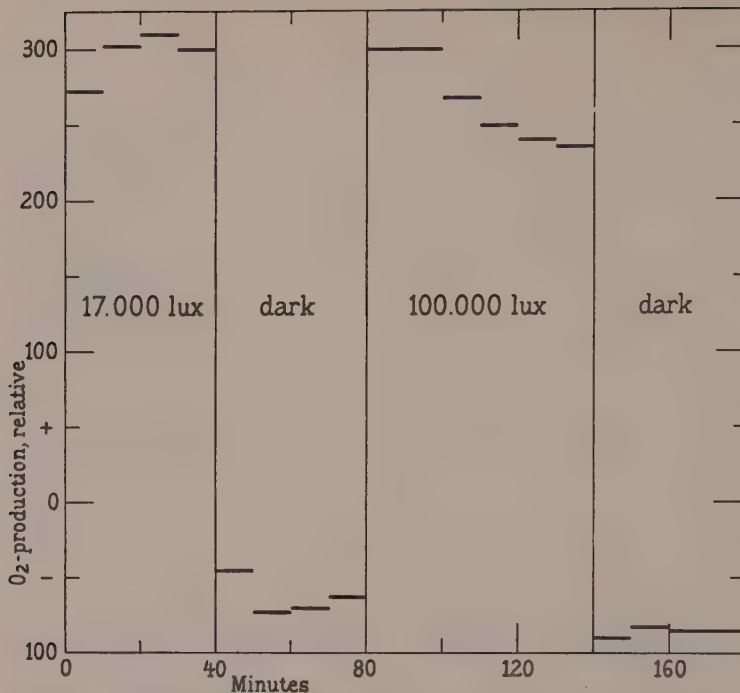


Fig. 7. Photosynthesis and respiration in *Cladophora intestinalis*.
18° C., pH 8. 24.8.1949.

producing optimum photosynthesis the rate of photosynthesis may still be limited by the chemical reactions. No further decrease in the rate of photosynthesis will then appear. This was the case during the experiments described in Figure 13 (Steemann Nielsen 1949).

The experiments described in Figures 5 and 6 give, however, diverging results. *Cladophora insignis* was used in these experiments, too. Here is found a marked further decrease in the rate of photosynthesis when changing from 100,000 lux to 35,000 or 17,000 lux. In these experiments the photochemical mechanism has been more seriously attacked than in the experiments described in the 1949 paper. It is to be assumed that the ratio photo-oxidation of enzymes : photo-oxidation of the photochemical mechanism varies.

Enzymes participating in respiration do not seem to be attacked. The rate of respiration was thus found to be a little less in experiments following illumination at 17,000 lux than in experiments following illumination for 60 minutes at 100,000 lux (Figure 7).

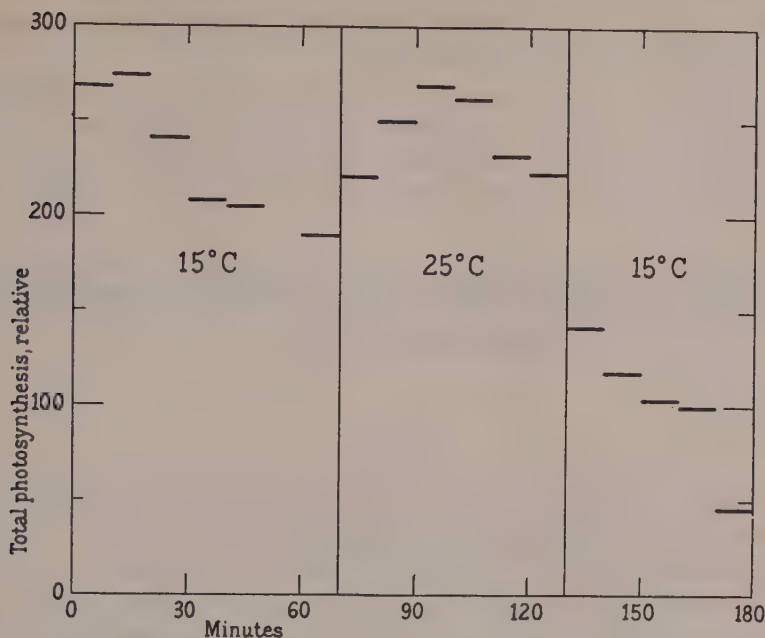


Fig. 8. Photosynthesis in *Cladophora intestinalis*. 15° C. or 25° C., pH 8. 30.8.1949.

It is thus to be assumed that only cell components found in the chloroplasts are attacked by the chlorophyll-sensitized photo-oxidation. The enzymes participating in normal respiration of the photosynthesizing cells are after all located outside the chloroplasts.

3. The Dependence of Temperature on the Rate of Photosynthesis in Extraordinarily Bright Light

It is well known that in bright light the rate of photosynthesis is dependent on temperature. As the rate in bright light is limited by the pace of the chemical reactions, this is quite natural. When in extraordinarily bright light the rate of photosynthesis is reduced through photo-oxidative decomposition of one or more of the enzymes in photosynthesis, the rate must still be dependent on temperature.

Figure 8 shows that this is really so. Two other series of experiments have rendered quite similar results. It is notable that the rate of photosynthesis (real assimilation) does not increase instantaneously when changing the temperature from 15° to 25° C. During the first 20 minutes at 25° C. the

rate steadily increased. After that the decrease started again according to the photo-oxidative destruction of the enzymes.

The 20 minutes required for establishing the higher rate of photosynthesis when changing to a higher temperature probably indicate that the long activation (or induction) periods often found in photosynthesis of water plants (cf. e.g. Steemann Nielsen 1942) is not caused by enzymes being mobilized.

According to Österlind (1951) the factor to be activated is either connected to the absorption of bicarbonate ions or is carbonic anhydrase. The last mentioned possibility thus can be eliminated.

Summary

In exceedingly bright light photo-oxidation besides enzymes attacks part of the photochemical mechanism in photosynthesis, too. It is rendered probable that the temporary inhibition of the rate of photosynthesis often occurring at a low light intensity after changing from a higher light intensity is also caused by photo-oxidation. Inactivation of the photochemical part of photosynthesis, therefore, is not assumed to be part of normal photosynthesis.

It is rendered probable that the long activation periods often found in photosynthesis of water plants is not caused by enzymes being mobilized.

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References

- Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. & Stepka, W.: The Path of Carbon in Photosynthesis V. Paper Chromatography and Radioautography of the Products. — *J. Amer. Chem. Soc.* 72: 1710. 1950.
- Myers, J. & Burr, G. O.: Studies on Photosynthesis. Some Effects of Light of High Intensity on *Chlorella*. — *J. Gen. Physiol.* 25: 45. 1940.
- Steemann Nielsen, E.: Der Mechanismus der Photosynthese. Versuche mit *Fucus serratus* und anderen Submersen. — *Dansk Bot. Ark.* 11, Nr. 2: 1. 1942.
- A reversible Inactivation of Chlorophyll in Vivo. — *Physiol. Plantar.* 2: 247. 1949.
- Stålfelt, M. G.: Licht- und Temperaturhemmung in der Kohlensäureassimilation. — *Planta* 30: 384. 1939.

A Comparative Study of the Breakdown of Cellulose by Microorganisms

By

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A scheme for the mechanism of cellulose breakdown has been postulated (Reese, Siu and Levinson 1950) wherein at least two steps are involved in the conversion of cotton cellulose to cellobiose or other substrates hydrolyzed by β -glucosidase. This mechanism has been deduced primarily from differences in the behavior of certain organisms on various cellulosic substrata. Attempts to elucidate the nature of the first step, C_1 , have been unsuccessful. Consequently, further comparisons of a much greater number of organisms was made in an effort to find those of possible value in clarifying the picture. For example, an organism capable of growing on highly degraded cellulose, but not on native cellulose, might be considered lacking in C_1 , but possessing all subsequent abilities. Such organisms have been found. Unfortunately, the unavailability of well-identified intermediate hydrolysis products, and the uncertainties regarding the structure of native cellulose itself, are major obstacles to a final solution of the problem.

The organisms occurring in the lists found in this paper represent but a small part of the total number studied. They have been chosen to represent a wide variety of organisms. Those that have been omitted are, in the main, species of *Aspergillus* and of *Penicillium*. The ability of organisms to grow upon selected substrata has been recorded, but most of the emphasis here is on the nature of the enzymes which are produced by the organism and detected in the surrounding medium.

Methods and Definitions

The methods employed in the current work were essentially the same as those previously reported. An organism is considered cellulolytic if it brings about a 10 per cent loss in tensile strength of cotton duck strips within a period of two weeks under the test conditions (Reese and Downing 1951). Solubilization of cotton by microorganisms is believed to entail at least two steps, the last of which is the hydrolysis of the β -1,4 linkages of the cellulose chain by an enzyme designated as Cx. Cx activity was measured against soluble cellulose derivatives of low degree of substitution as:

- (a) Increase in reducing sugars (Reese *et al.* 1950), or
- (b) Change in fluidity (Levinson and Reese 1950)

Carboxymethyl cellulose, Na salt (CMC) has usually served as the substratum. Results are obtainable within one hour. While data in terms of reducing sugars produced in mg/ml/hr are adequate for most comparisons, for more accurate work either changes in fluidity per unit time (d/dt) or Cx units are used. A Cx unit is defined as that amount of enzyme which catalyzes the production of 0.40 mg of reducing sugar as glucose per ml of reaction mixture per hour under the conditions of the test (50°C ; pH 5.4; CMC 50T = 0.5 per cent).

Media of comparatively low salt content were used in all of our work. Generally the medium for Cx production consisted of NH_4NO_3 0.1 per cent + KH_2PO_4 0.14 per cent + $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02 per cent, + yeast extract (Difco) 0.01 per cent, + cellulose 0.4–0.5 per cent. The pH was adjusted with NaOH to 6.1–6.3. After inoculation and incubation on a shaker, the insoluble solids, including mycelium, were removed from the culture by filtration through a medium porosity fritted glass crucible. The filtrates were preserved for further use by addition of merthiolate. The pH was adjusted with citrate buffer to about 5.6 and the filtrate stored in a refrigerator.

All hydrolytic enzymes were assayed under similar conditions of time, temperature and pH. For pectinase determinations, 0.15 per cent pectic acid adjusted to pH 5.2–5.4 was used as substratum. The reducing material resulting from hydrolysis is estimated from a glucose curve, and while this is not a quantitative measure of the end products, it does give figures useful for comparison of relative filtrate activity.

Polyphe nol oxidase activity was detected by adding 8 drops of a 1 per cent guaiacol solution to 2 ml of test filtrate buffered with citrate to about pH 5.5. Incubation was at room temperature for 4 hours. If no pink color developed in this time, the oxidase was considered absent. A relative index of activity is the time required for pigment formation, the shorter the time, the higher the activity.

The following substrata have been used in this work:

- (a) Cotton sliver, long staple from Cramerton Mills. This has been dewaxed with benzene-alcohol, and ground to 40 mesh in a Wiley Mill.

(b) Wood cellulose

1. Solka Floc: Brown Co., N.Y.C., (from spruce by sulfite process) 94 % cellulose, 2.6 % pentosan, 0.1 % lignin.
 2. RCB 80: W. Va. Pulp & Paper Co. (from oak) 84 % cellulose, 18 % pentosans, 0.6 % lignin — According to company literature and communications, a modified soda cooking process was used, followed by a four stage bleaching system consisting of chlorine bleach, caustic extraction, and two calcium hypochlorite stages.
 3. Filter paper: Whatman No. 1 ground in Wiley Mill.
- (c) Carboxymethyl cellulose, Na salt CMC 50T: from Hercules Powder Co. Medium viscosity; degree of substitution ca 0.52.
- (d) Alkali cellulose: Purified cotton linters (Hercules Powder Co.) was treated with 35 per cent NaOH for 30 minutes, filtered and washed thoroughly. There was no exposure of the fibers to air before the washing. Oxidation effects may be considered near minimum.

Results

I. *Distribution of Cx Activity Among Microorganisms.* It was previously shown (Reese *et al.* 1950) that Cx is produced by cellulolytic and by some non-cellulolytic organisms. More recently, it was found possible to detect Cx in culture solutions of tests involving loss in tensile strength of duck strips. Since part of our program involves routine testing of organisms for ability to degrade duck, an abundance of material was available for correlating loss in tensile strength with presence of Cx in the solution. Under the test conditions — i.e. duck strips one-half immersed in mineral salts solution in test tubes — reducing sugar often accumulates in active cultures. This situation rarely occurs in shake flasks where there is an adequate air supply.

As a general rule, Cx activity is high in cultures actively degrading cotton duck, and low in those cultures producing little or no loss in tensile strength of the duck (Table 1). There are conspicuous exceptions, however, and these require careful analysis. In some cases (i.e. *Humicola fuscoatra*, etc.) high loss in tensile strength is accompanied by low Cx activity in the filtrate. Differences in adsorbability of Cx's of various origins have been found (Table 3). Removal of Cx from solution by adsorption on the cellulose of the duck strip may account for low Cx values accompanying some high losses in tensile strength. Perhaps, in terms of our current hypothesis, it is C₁ and not Cx, that is responsible for the tensile strength loss. Cx activity coming later is necessary to supply the fragments capable of entrance into the cell, fragments that are required for growth and further C₁ production. We do not, however, refuse to acknowledge that there are other possibilities as (a)

Table 1. Activity of Microorganisms on Cotton Duck as Determined by Loss in Tensile Strength of the Duck and Cx Activity of the Medium.

Table 1 a. Cellulolytic Organisms.

Organisms	QM No.	Incub. Days	Loss in T.S. of Duck, %	Activity ¹ of Filtrate	
				Cx	β gluc.
Actinomycetes					
<i>Actinomyces</i> sp.	B 814	14	71	0.28	0.00
Bacteria					
<i>Cellulomonas fimi</i>	B 527	12	44	0.06	0.00
<i>Cellulomonas</i> sp.	B 1535	12	27	0.01	0.00
<i>Cellvibrio fulvus</i>	B 18	12	60	0.18	0.00
<i>Cellvibrio vulgaris</i>	B 122	12	76	0.71	0.00
<i>Corynebacterium</i> sp.	B 515	12	42	0.17	0.00
<i>Sporocytophaga myxococcoides</i>	B 482	12	100	0.40	0.00
Chytrid					
<i>Karlingia rosea</i>	517	14	100	0.62	0.00
Ascomycetes					
<i>Chaetomium causiaeformis</i>	949	14	100	0.15	0.00
<i>Chaetomium globosum</i>	459	14	100	0.50	0.00
<i>Chaetomium turgidopilosum</i>	948	14	32	0.34	0.00
Basidiomycetes (se also Table 5)					
<i>Coprinus sclerotigenus</i>	933	14	89	0.28	0.00
Fungi Imperfecti					
<i>Aspergillus fumigatus</i>	45 h	14	82	0.80	0.00
<i>Aspergillus luchuensis</i>	873	14	27	0.60	0.09
<i>Aspergillus nidulans</i>	87 c	14	53	0.80	0.08
<i>Aspergillus niger</i>	877	14	38	0.75	0.01
<i>Aspergillus rugulosus</i>	886	14	50	0.88	0.06
<i>Arthrobotrys superba</i> v. <i>oligospora</i> ..	671	14	96	0.25	0.00
<i>Botrytis cinerea</i>	520	14	94	0.42	0.00
<i>Cladosporium herbarum</i> 30° C.	1027	21	0	0.00	—
<i>Cladosporium herbarum</i> 25° C.	—	21	71	0.67	—
<i>Coccospora agricola</i>	336	14	94	0.54	—
<i>Fusarium</i> sp.	967	14	93	1.01	0.00
<i>Humicola fuscoatra</i>	73 d	14	100	0.16	0.00
<i>Humicola grisea</i>	542	14	100	0.38	0.00
<i>Monilia sitophila</i>	909	14	81	(0.21) ²	—
<i>Myrothecium verrucaria</i>	460	14	100	1.01	0.09
<i>Papulaspora</i> sp.	973	14	63	0.38	—
<i>Penicillium luteum</i>	474	14	96	0.69	0.01
<i>Penicillium capsulatum</i>	J 1264	14	24	0.85	0.09
<i>Pestalotia palmarum</i>	381	14	100	1.08	0.39
<i>Phoma</i> sp.	926	14	87	0.88	0.00
<i>Scopulariopsis brevicaulis</i>	815	14	70	0.61	0.00
<i>Torula</i> sp.	986	14	100	0.97	0.06
<i>Trichoderma viride</i>	6 a	14	66	0.18	—

¹ Activity expressed in terms of reducing sugar as mg glucose per ml per hour under test conditions (Reese, Siu, Levinson 1950).

² after autoclaving

Table 1 b. *Non Cellulolytic Organisms.*

Organisms	QM No.	Incub. Days	Loss in T.S. of Duck, %	Activity of Filtrate	
				Cx	β gluc.
Bacteria					
<i>Micrococcus</i> sp.	B 30	12	0	0.00	
<i>Proteus vulgaris</i>	B 1464	12	0	0.00	
<i>Pseudomonas</i> sp.	B 1482	12	0	0.00	
Phycomycetes					
<i>Choanephora cucurbitarum</i>	1042	14	1	0.00	
<i>Circinella sydowi</i>	629	21	0	0.05	
<i>Conidiobolus villosus</i>	929	14	0	0.00	
<i>Cunninghamella bertholletiae</i>	1021	14	10	0.00	
<i>Mucor hiemalis</i>	775	28	5	0.04	
<i>Mycotypha microspora</i>	966	14	13	0.06	
<i>Rhizopus arrhizus</i>	808	28	2	0.00	
<i>Sporodinia grandis</i>	1063	14	2	0.00	
Basidiomycetes					
<i>Ustilago zeae</i>	990	14	1	0.02	
Fungi Imperfecti					
<i>Acremoniella atra</i>	1045	14	1	0.03	
<i>Isaria tenuipes</i>	1004	14	0	0.00	
<i>Microsporum</i> sp.	912	14	0	0.00	
<i>Monotospora lanuginosa</i>	225	14	2	0.02	
<i>Paecilomyces varioti</i>	10 a	28	1	0.00	
<i>Penicillium funiculosum</i>	124a-2	14	0	0.00	
<i>Penicillium lilacinum</i>	4 e	14	0	0.00	
<i>Pullularia pullulans</i>	279 c	28	0	0.00	
<i>Sepedonium chrysospermum</i>	1055	14	0	0.00	
<i>Spicaria violacea</i>	1034	14	1	0.04	

Table 1 c. *Organisms Producing Cx — Yet Showing Little or no Loss in T. S. of Duck.*

Organisms ¹	QM No.	Incub. Days	Loss in T.S. of Duck, %	Activity of Filtrate	
				Cx	β gluc.
<i>Aspergillus flavus</i>	10 e	28	14	0.82	0.41
<i>Aspergillus sydowi</i>	31 c	28	0	0.29	0.00
<i>Aspergillus tamarii</i>	50 b	21	0	0.48	—
<i>Penicillium chrysogenum</i>	51 b	28	9	0.23	0.12
<i>Penicillium frequentans</i>	409	14	3	0.54	—
<i>Penicillium spinulosum</i>	108 b	14	3	0.34	
<i>Penicillium turbatum</i>	138 h	14	1	1.01	0.00

¹ additional organisms of this type are listed in table 5.

greater localization of action (b) inactivation of enzyme, (c) differences in growth curves and autolysis.

In other cases, organisms grow very little on the cotton duck, cause little or no significant loss in tensile strength of the duck, yet produce remarkably high Cx values in the filtrate (Table 1 c). An outstanding example is *Penicillium pusillum* Smith, QM 137 g. Whereas under the experimental conditions, strong cellulose destroyers (*Myrothecium verrucaria*, etc.) produce up to 5 Cx units per ml and bring about a complete loss in tensile strength of duck (in 7 days), *P. pusillum* Smith may produce 100 Cx units while reducing the tensile strength (in 28 days) by less than 30 per cent. Thus a weakly cellulolytic organism is found to produce 20 times the amount of one member of the cellulase complex (i.e. of Cx) than is ever produced by the most active cellulose degraders. While this observation has been made many times, much remains to be cleared up. This same *P. pusillum* Smith grown on ground duck in shake flasks for the same period of time produced a maximum of 4.0 units Cx/ml. This is of the same magnitude as that obtained from active cellulolytic organisms under identical conditions. The major differences between the two set-ups (i.e. shaker vs. test tube) appear to be (a) degree of aeration, (b) amount of substrate per unit volume of liquid (greater in test tubes), and (c) sporulation, the latter occurring only in test tube culture. However, *M. verrucaria* and other active destroyers of cellulose produce approximately equal amounts of Cx in shake flasks and in test tubes, but active sporulation occurs only in the latter.

In a wide survey of organisms growing on duck strips in test tubes, the following were found to produce filtrates containing the highest concentration of Cx:

1. <i>Penicillium pusillum</i> Smith	QM 137g	180 Cx μ /ml
2. <i>Penicillium capsulatum</i>	J 1264	35 Cx μ /ml
3. <i>Penicillium soppi</i>	QM 976	35 Cx μ /ml
4. <i>Pestalotia palmarum</i>	QM 381	40 Cx μ /ml

It must be pointed out that these data represent maximum potencies in dilution units and are thus not directly comparable to the data (Table 1) in which only the full strength solution was tested and the results expressed in mg of reducing sugar/ml/hr. Maximum yields are often reached only after prolonged incubation periods. Whereas comparisons on the basis of mg/ml/hr are valid at low Cx values, when the reducing value as glucose exceeds 0.5—0.6 mg/ml the proportionality has less meaning. Values of this kind are taken in screening operations where large number of determinations are made. For a better picture of comparative activity, dilution units are of far more value.

Attention is called to other items of interest (Table 1):

- (a) *Sporocytophaga myxococcoides* is among those actively cellulolytic organisms producing Cx. In order to demonstrate the presence of this enzyme, extracellularly, the cells had to be removed. This was readily accomplished by the addition of aluminum chloride to the solution.
- (b) *Cladosporium herbarum*, as previously shown by Marsh, Bollenbacker, Butler & Raper (1949), is very sensitive to temperature. Cellulolytic activity is apparent at 25° C but not at 30° C.
- (c) *Aspergillus flavus* seldom shows as great a loss in tensile strength as the 14 per cent in 28 days in the test recorded.

The exceptional nature of the extreme cases cited above are of interest in themselves, and because they may prove to be useful in elucidating the mechanism of cellulose breakdown. However, they should not be so over-emphasized that one loses sight of the fact that Cx is *always* produced in appreciable amounts by microorganisms engaged in the hydrolysis of cotton duck in test tubes. A great many more organisms than those listed (Table 1 a) have been tested and this has never failed to be the case.

II. *Effect of Substratum on Cx Production in Shake Flasks.* Approximately 0.4 per cent of ground cellulose was incorporated in a yeast extract — mineral salts medium and incubated in shake flasks with the test organism. At intervals during a two week period the culture medium was filtered and assayed for Cx. In most cases, the data (Table 2) are results of several experiments from which the highest values have been recorded. Cx values below 0.05 mg/ml/hr are of doubtful significance, since these approach the limits of usefulness of the dinitrosalicylic acid method for the determination of reducing sugars.

Based on behavior in *shake flasks*, the organisms may be arranged (Table 2) into 5 groups:

- Group A — Cellulolytic. Produces Cx in the absence of cellulose or its derivatives.
- Group B — Cellulolytic. Produces Cx on a wide variety of cellulosic substrata, but not from non-cellulosic materials.
- Group C — Cellulolytic. Produces Cx from many cellulosic substrata, but none is detectable from alkali linter cellulose — although usually the alkali cellulose is rapidly broken down by the fungus.
- Group D — Cellulolytic. Little or no Cx accumulates in the medium on any cellulosic substratum.
- Group E — Non-cellulolytic.

Table 2. *Effect of Substratum on Cx Production by Microorganisms in Shake Flasks.*

Organisms	QM No.	Cx Activity mg/ml/hr						% Loss in T.S. (Duck; 2 wks.)
		Glucose	Cellulose		Modified Cellulose			
			Cotton	Wood	HEC	Dextrin	Alk. Cell.	
Group A								
<i>Aspergillus luchuensis</i>	873	0.66	0.36	0.51	0.20	0.09	0.06	42
Group B								
<i>Actinomyces</i> sp.	B 814	0.00	0.60	0.98	0.98	0.67	0.55	58
<i>Aspergillus fumigatus</i>	45 h	0.03	0.67	0.43	0.12	0.31	0.32	72
<i>Coprinus sclerotigenus</i>	933	0.00	0.24	0.44	0.10	0.16	0.80	85
<i>Myrothecium verrucaria</i>	460	0.04	0.95	1.04	0.33	0.49	0.93	100
<i>Penicillium luteum</i>	474	0.04	0.63	0.47	0.59		0.39	96
<i>Schizophyllum commune</i>	812	0.01	0.33	0.61	0.37		0.60	76
<i>Sporotrichum pruinosum</i>	168	0.00	0.71	0.59	0.83		0.14	90
<i>Torula</i> sp.	986	0.09	0.13	1.02	0.08		0.62	100
Group C								
<i>Aspergillus niger</i>	877	0.00	0.27	0.90	0.08		0.00	38
<i>Chaetomium indicum</i>	46 b	0.00	0.13	0.79	0.66		0.07	100
<i>Fusarium roseum</i>	38 g	0.01	0.38	0.63			0.02	90
<i>Fusarium</i> sp.	967	0.02	0.54	0.10	0.25		0.05	93
<i>Karlingia</i> sp.	517	0.03	0.46	0.28	0.56		0.01	100
<i>Memnoniella echinata</i>	1 c	0.00	0.48	0.39	0.09	0.00	0.13	100
<i>Sporocytophaga myxococcoides</i>	B 482			0.30		0.01	0.14	100
<i>Trichoderma viride</i>	6 a	0.00	0.89	0.86	0.00	0.45	0.00	90
Group D								
<i>Botrytis cinerea</i>	520	0.00	0.03	0.03	0.09	0.00	0.03	60
<i>Cellovibrio fulvus</i>	B 18			0.03	0.00	0.00	0.00	60
<i>Cellvibrio vulgaris</i>	B 122			0.00	0.17	0.00	0.00	76
<i>Chaetomium globosum</i>	459	0.00	0.03	0.09	0.08		0.10	100
<i>Scopulariopsis brevicaulis</i>	815	0.00	0.00	0.05	0.05		0.04	100
Group E								
<i>Aspergillus flavus</i>	10 e	0.00	0.03	0.51	0.13	0.00	0.06	0; 4; 14
<i>Aspergillus niger</i>	458	0.01	0.04	0.75	0.00	0.00	0.00	3
<i>Aspergillus sydowi</i>	31 c	0.00	0.04	0.34	0.18	0.01	0.13	G, ¹ G, 6
<i>Cladosporium herbarum</i>	489		0.00		0.00	0.00	0.00	G
<i>Mucor hiemalis</i>	775	0.00	0.00		0.00			5
<i>Paecilomyces varioti</i>	10 a	0.00	0.00		0.00		0.02	G; 1
<i>Penicillium frequentans</i>	J 1173	0.00	0.17	0.49	0.38	0.25	0.13	2 (3 wks.)
<i>Penicillium spinulosum</i>	213	0.00	0.09	0.35	0.26	0.31	0.00	6 (3 wks.)
<i>Pullularia pullulans</i>	279 c	0.00	0.00	0.00	0.00	0.00	0.00	0; 0
<i>Rhizopus arrhizus</i>	808	0.04			0.00			2
<i>Ustilago zeae</i>	990		0.00	0.00				1

¹ G indicates a slight increase in tensile strength.

Aspergillus luchuensis QM 873 — a member of the *A. niger* group — is the only member of group A. To date, we have detected Cx production by this organism not only on cotton duck, wood cellulose, and soluble cellulose derivatives, but also on cellobiose, glycerol, starch, and glucose. In fact, the latter two substrata have usually given the highest Cx concentrations. Hydro-

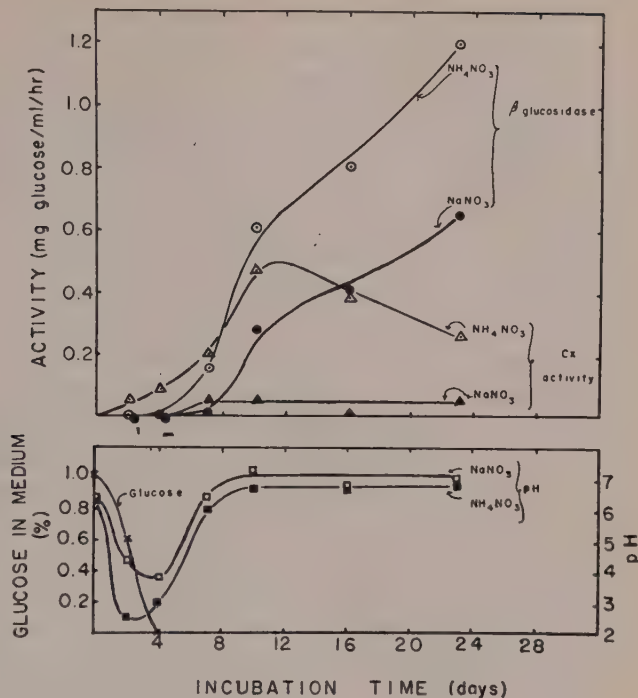


Fig. 1. Effect of N-Source on enzyme production by *Aspergillus luchuensis* QM 873.

Lower curves show the effect (1) on rate of utilization of glucose and (2) on pH.

gen ion concentration appears to be of some importance in Cx production by this organism.

The production of Cx and of β -glucosidase by this organism using NH_4NO_3 and NaNO_3 as nitrogen sources and dextrose as a carbon source was studied (Figure 1). With the exception of the early production of a small amount of Cx, the extracellular appearance of these enzymes was noted only after the glucose had been consumed and the pH had begun to rise. Concurrently with the rise in pH, there was a rapid increase in β -glucosidase concentration, a somewhat higher yield being obtained when NH_4NO_3 was used as the N-source than when NaNO_3 was used. The β -glucosidase production continues to increase even after the pH has reached constant level of 6.8–7.2. The Cx in the medium using NH_4NO_3 also increased fairly rapidly after the pH rise had begun, but fell off as the pH became constant. On NaNO_3 practically no Cx was found. Other experiments, using dextrose and NH_4NO_3 , showed that when the medium is buffered with chalk no Cx accumulates although β -glucosidase does. Inactivation of Cx at pH levels greater than 8.0 has been demonstrated in other experiments. The higher pH levels reached may be sufficient to account for the absence of Cx.

When nitrogen levels are increased, the enzyme production also increases. If the dextrose concentration is increased, without an increase in nitrogen concentration, the pH fails to rise after its first dip, and neither enzyme is detectable. It is seen (Figure 1) that β -glucosidase exceeds Cx production, (as measured in our assay) when *A. luchuensis* is grown on dextrose, but the reverse is true when the fungus is grown on cotton duck (Table 1). Finally, unpublished data show that in addition to Cx, amylase is produced by *A. luchuensis* from all substrata tested. Thus we have an organism, which is unique in our experience, in that it produces both α - and β -1,4 polysaccharases in the absence of the substratum for either.

In groups C and D are organisms that for the most part are active cellulolytic fungi (i. e. they rapidly lower the tensile strength of cotton duck), but which under some circumstances do not produce Cx in the outer solution. For some fungi, alkali cellulose is the only substrate with which Cx cannot be found, even though good growth and decomposition have taken place. For these, it must be concluded that Cx does play a role, but that its failure to accumulate in the medium is due either to adsorption or to inactivation, since all of the organisms of these groups produce an appreciable quantity of Cx when grown on duck strips in test tubes (Table 1). For example, *Chaetomium globosum* is an active cellulose degrader in test tubes producing appreciable amounts of Cx, yet in several shake flask experiments no Cx could be detected extracellularly although marked degradation (weight loss) took place. Further shaker tests offered a partial explanation for this. Within 6 days after inoculation, there was a loss in weight of the duck of 25 per cent, yet over a period of 16 days, the Cx activity of the medium was negligible (i.e. less than 0.04 mg/ml/hr). In the present test, incubation was prolonged, and the Cx values were found to increase. At 30 days, the Cx value was 0.24 mg/ml/hr and rose to 0.46 in 80 days. Thus, the presence of Cx is demonstrable during cellulose hydrolysis in shake flasks, even in those cases where casual observation might have indicated its absence.

Cx differs from organism to organism in adsorbability on cellulose. While none of the Cx of *Aspergillus niger* QM 877 was adsorbed on ground cotton sliver (Table 3), 48 per cent of the Cx of *Trichoderma viride* was adsorbed. Adsorption thus may account for those examples of high rates of decomposition of cotton in which the Cx content of the solution is low. Chemical treatment of the cotton also modifies its adsorptive properties (Table 3). The mild boiling of cotton with 1 N alkali for 15 minutes roughly doubles the adsorption of Cx, while boiling with 1 N HCl has no effect although this treatment reduces the degree of polymerization and increases the per cent crystallinity. The greatest increase in adsorption was obtained by the cotton pre-treated with 35 per cent NaOH (our »alkali cellulose«). In our com-

Table 3. Comparison of Organisms on Basis of Cx Adsorption on Cotton.

Organisms	QM No.	0/o Cx Adsorbed by Cotton			
		Untreated	15 min. Boil N/1 NaOH	35 0/o NaOH	15 min. Boil N/1 HCl
<i>Aspergillus fumigatus</i>	45 h	10	15	35	10
<i>Aspergillus niger</i>	877	0	0	0	0
<i>Myrothecium verrucaria</i>	460	12	33	55	15
<i>Myrothecium verrucaria</i>	(Retest)	22	40	64	14
<i>Penicillium pusillum</i> Smith	137 g	0	17	67	13
<i>Scopulariopsis brevicaulis</i>	815	(— 4)	4	43	(— 8)
<i>Sporotrichum pruinosum</i>	168	4	8	24	4
<i>Trichoderma viride</i>	6 a	48	96	94	60

Conditions: 10 ml enzyme solution of 1.0 Cx μ /ml potency in citrate pH 5.4; 90 mg substrate; shake 30 minutes at 30° C, and then test supernatant for Cx activity.

parison of organisms (above), group C was characterized by a low Cx concentration in the medium when the fungus was grown on alkali cellulose as compared to much higher concentrations when other cellulose sources were used. This observation is supported by the much higher affinity of Cx for alkali cellulose shown here. Differences in pH further widen the differences in the per cent of Cx adsorbed, adsorption increasing with increasing hydrogen ion concentration (at least down to pH 2.5, unpublished data). This, too, may partially explain why Cx is invariably found in the test tube experiments. In tests of this sort, the pH usually remains above 5.5, while in shaker tests the pH may drop below 4.0.

A careful watch has been kept for organisms which might preferentially utilize cotton cellulose to wood cellulose as a substratum for growth. In single experiments, such a preference has occasionally been observed. Indeed *Fusarium* sp. QM 967 and *Memnoniella echinata* QM 1 c at first appeared to be in such a category. Further examination revealed that the results were due to a more rapid lowering of the pH during growth on wood cellulose. When chalk or other means of keeping the pH up were employed, there was essentially no difference in availability of the two substrata. Our results to date indicate that where an organism shows a preference, it is the wood cellulose which is the substratum of choice.

The »non-cellulolytic« microorganisms (group E) are unable to degrade cotton cellulose. Most of these are unable to hydrolyze either cellulose or its derivatives. But as a result of continual testing with variation in substrata and procedure, there have appeared a few organisms of doubtful complexion. As previously shown (Reese and Downing 1951), all *Aspergilli*, whether cellulolytic or not, can hydrolyze some soluble cellulose derivatives, thus

possessing the ability to produce Cx. Our criterion for »cellulolytic» has been ability to produce loss in tensile strength of cotton. Now it has been found that *A. flavus*, *A. niger* QM 458 and others which produce little or no loss in strength of duck, grow well on wood cellulose containing pentosan, and release Cx into the medium in appreciable amount. Reexamination of the duck-strip data (Tables 1 and 5) shows that *A. flavus* and many other organisms that do not cause loss in tensile strength do produce a little growth and varying amounts of Cx. The brown rot fungi to be considered later behave similarly on cotton duck. These organisms of group E are interesting because some of them are truly borderline with respect to their cellulolytic ability (Brown rotters; *Asp. flavus*; *A. sydowi*; *A. tamarii* etc.):

1. On cotton duck strips, none brings about a loss in tensile strength but still a small amount of growth occurs and an appreciable amount of Cx appears in the medium.
2. On some soluble cellulose derivatives of low degree of substitution, growth occurs and Cx appears in the medium.
3. On wood cellulose (RCB 80) in shake flasks, growth takes place and the losses in weight exceed by far the amount of pentosan present. Cx appears in the filtrates.
4. On cotton in shake flasks, no growth takes place nor is Cx produced. Ground dewaxed fiber, purified linters, and ground duck have all been tested. Linters swollen with 35 per cent NaOH, and a wood cellulose low in pentosan 2.6 per cent (Solka Floc) are also resistant.

Aspergillus niger QM 458 is an organism widely used in deterioration studies. It is a strain originating in the USDA (TC 215-4247) and distributed by the American Type Culture Collection as No. 6275. It has always been considered non-cellulolytic (Marsh *et al.* 1949 and QM reports), and must continue to be so considered based on its inability to degrade cotton. With the discovery that other »non-cellulolytic» *Aspergilli* are able to degrade wood cellulose (RCB 80) and to produce Cx, we attempted to grow this *A. niger* QM 458 under similar conditions. After a slow start, growth was apparent in the shake flasks. At the end of 4 weeks there was a 50 per cent weight loss, equivalent to a somewhat higher but indeterminate loss in substratum since part of the residue is mycelium. Allowing for the pentosan (18 per cent) present in the wood cellulose, it is apparent that much cellulose has been degraded. The Cx activity reached 0.59 mg/ml/hr. Present indications are that »non-cellulolytic» organisms which grow on the soluble cellulose derivatives will grow on this wood cellulose. It should be made clear that in this work two strains of *A. niger* have been used. The »non-cellulolytic» QM 458 (above) is representative of most of the isolates of the truly black *Aspergilli*. One

»cellulolytic» strain has been found (QM 877), which is like the other strains except that it can lower the tensile strength of duck.

In concluding this section, attention should be called to the fact that nearly all phycomycetes are non-cellulolytic. Exceptions are found among the chytrids, the Saprolegniaceae (Bhargava 1943), and the Peronosporaceae (Mehrotra 1949 for species of *Phytophthora*). All attempts to grow our representatives, most of which belong to the Mucoraceae, on cellulose of various sources have failed. One organism in particular, *Rhizopus* sp. QM 1032, has been subjected to various tests because it is well known as producing a boll rot in cotton (Ray 1946). Using unopened bolls (kindly supplied by Dr. Paul Marsh USDA), we can completely affirm that this organism grows abundantly on autoclaved, or on surface sterilized bolls. It grows well, too, on the contents of the locules removed under sterile conditions. Under no circumstances, however, have we been able to get the organism to grow on the cotton, free of the seeds and oil. Tested on several cellulosic materials, this organism has never showed signs of growth nor has the enzyme Cx ever been detected in the filtrates. Other tests have shown that *Rhizopus* sp. QM 1032 grows very well on coconut oil. Its growth in the boll may be at the expense of the cottonseed oil, or perhaps on the soluble reducing compounds observed by Marsh (1951).

Cx, like the pectic enzymes reported by Phaff (1947), is an adaptive enzyme in most fungi. But unlike the pectic enzymes, Cx production is not stimulated by the products of the reaction, i.e. by cellobiose. In our experience, *Aspergillus luchuensis* is the only organism of 28 tested (Table 2) which can produce Cx in the absence of cellulose, or of cellulose derivatives. Even in *A. luchuensis* where Cx is produced during growth on cellobiose, the reaction is non-specific for cellobiose, since glycerol, as a carbon source also leads to Cx production.

III. *Role of β -glucosidase in Utilization of Cellulose by Microorganisms.* We have shown earlier that little or none of the β -glucosidase, cellobiase, is present extracellularly during cellulose hydrolysis by most microorganisms. Norkrans (1950) found a similar situation in *Tricholoma* sp. Bhargava (1943) and Mehrotra (1949) were able to demonstrate extracellular β -glucosidase (amygdalin substratum) in the Saprolegniaceae and in *Phytophthora* spp., but in much lower amounts than found intracellularly. Their incubation time was 7 days at 37° C, as compared to our 1 hour at 50° C, so that it is likely that the absolute concentration of enzyme found extracellularly was extremely low. Ploetz (1939) demonstrated cellobiase in the sap pressed out of *Merulius lacrymans*, but this is intracellular material and is not to be confused with extracellular activity.

Table 4. *Organisms Producing β -glucosidase in Addition to Cx When Grown on Duck Strips.*

Organisms	QM No.	Filtrate Activity ¹ Against		% Loss in Tensile Strength 4 wk.	Ratio Salicin Activity Cellobiose Activity
		CMC 4 wk.	Salicin 4 wk.		
<i>Aspergillus flavus</i>	10 e	0.11	0.25	0	2.2
<i>Aspergillus luchuensis</i> ...	873	0.60	0.14	50	3.6
<i>Aspergillus niger</i>	877	0.80	0.20	67	0.62
<i>Aspergillus phoenicis</i>	1005	0.75	0.30	63	0.55
<i>Penicillium janthinellum</i> ...	Fla D 87	0.93	0.30	91	3.1
<i>Penicillium piscarium</i> ...	96 e	0.76	0.34	81	3.2
<i>Penicillium soppi</i>	976	1.0 +	0.40	79	2.8
<i>Penicillium soppi</i>	63 a	0.86	0.28	100	2.7
<i>Pestalotia palmarum</i>	381	1.0 +	0.62	100	2.2

¹ Activity expressed as mg glucose/ml/hr at 50° C.

All β -glucosidase values were determined using salicin as substratum at a concentration of 0.0043 M. Comparisons have been made from time to time with cellobiose as substratum at an equimolar concentration. Glucose formed from the latter was oxidized with glucose oxidase as earlier described (Levinson *et al.* 1951). *Aspergillus fumigatus*, *Trichoderma viride*, *Actinomyces sp.*, and *Karlingia rosea* are active cellulose destroyers representing widely different types of organisms. When grown on cellulose, these organisms produced filtrates which are completely lacking in β -glucosidase. Filtrates of other fungi grown on starch, or on dextrose, contain β -glucosidase. The rate of hydrolysis of salicin by these filtrates is two to three times the rate of hydrolysis of cellobiose.

As a result of a routine screening of large numbers of cultures (Table 1), several instances have been found where the amount of β -glucosidase in the medium is appreciable (Tables 1 and 4). That these cases are from duck strip tests, and that the organisms are *Aspergilli* and *Penicillia*, is due to the current nature of the screening program with emphasis on these two genera. The filtrates of these organisms were compared for relative activity on cellobiose and on salicin. The results are in agreement with those previously obtained i.e. salicin was hydrolyzed 2—3 times faster than cellobiose by all filtrates except those of the black *Aspergilli* in which case cellobiose was hydrolyzed at twice the rate of salicin (Table 4). It is interesting that all 4 black *Aspergilli* should differ from all other organisms tested. Two of the four are weakly cellulolytic (*A. niger* QM 877, *A. phoenicis* QM 1005), the others »non-cellulolytic» (*A. niger* QM 458, *A. carbonarius* QM 331).

It is customary to assume that organisms produce enzymes that hydrolyze naturally occurring substrata more readily than they hydrolyze similar but

unfamiliar substances. While salicin is found in nature it is comparatively rare, especially when compared to cellulose, and thus to the cellobiose occurring during the hydrolysis of cellulose.

It is interesting that in all the cases studied the β -glucosidases of strongly cellulolytic organisms should be more active on salicin than on cellobiose, while the β -glucosidase of a non-cellulolytic organism (*A. niger* QM 458) grown on starch should be more active on cellobiose than on salicin. The presence of a glucose oxidase in the filtrates of true black *Aspergilli* could explain the low results for salicin hydrolysis since the glucose formed would be oxidized. Actual tests, however, revealed the complete absence of glucose oxidase in the filtrates of the black *Aspergilli*, or of any of the other organisms tested. The other possibility, i.e. that a cellobiose oxidase is present, has likewise been eliminated (manometric tests by G. R. Mandels). It may be, that as with the invertases, there are two distinct types of hydrolytic enzymes, one acting on the glucone, the other on the aglucone portion of the molecule.

From the above, it is apparent that β -glucosidase values obtained using salicin as substratum may be generally too high as a measure of cellobiose activity. However, these values do serve to demonstrate the relatively insignificant amounts of cellobiose present extracellularly.

Aspergillus luchuensis, QM 873, also produces some β -glucosidase. Grown on non-cellulosic substrata, this fungus is the best source of β -glucosidase (Figure 2) we have found. Where traces of Cx are present in a solution, they may be removed by passing the solution, adjusted to pH 3.5, through a cellulose column. Both Cx and β -glucosidase are adsorbed but only the latter is eluted with 0.1 per cent NaHCO_3 . The eluates are useful in the analysis of cellobiose.

It is apparent, then, that β -glucosidase is absent extracellularly when most organisms attack cellulose. Even when cellobiose was used as substratum, β -glucosidase was absent extracellularly (in most cases). An interesting correlation was found between groups of organisms and their utilization of cellobiose. The non-cellulolytic phycomycetes (*Rhizopus oryzae*, *R. arrhizus*, *Cunninghamella elegans*, *Zygorrhynchus moelleri*, *Circinella sydowi*, *Mucor genevensis*) grew poorly on cellobiose, consuming about one half the total sugar in two weeks. Both cellulolytic and non-cellulolytic members of the Fungi Imperfecti consumed all of the cellobiose in the same incubation period.

IV. *Wood Rotting Fungi: Type of Rot vs. Activity on Cotton Duck.* The wood rotting fungi fit pretty well into the picture developed for other cellulolytic organisms. All produce extracellularly the enzyme Cx when grown on cellulose (Table 5), and none produces more than trace amounts of β -glucos-

Table 5. *Type of Rot vs Ability to Degrade Cotton Duck.*

Organisms	QM No.	Basal Medium		Basal + malt extract (0.05 0/0)		Basal + malt (0.05 0/0) + glu-cose (0.05 0/0)	
		0/o Loss T. S.	Cx	0/o Loss T. S.	Cx	0/o Loss T. S.	Cx
White rots:							
<i>Collybia velutipes</i>	1012	30	0.14	56	0.78	65	1.03
<i>Corticium vagum</i>	(ATCC 6221)	55 *	(Marsh' data)				
<i>Fomes annosus</i>	1018	11	0.40				
<i>Pholiota adiposa</i>	512	76 *	0.29 *	85	0.56		
<i>Pleurotus ostreatus</i>	987	100	0.33				
<i>Polyporus versicolor</i>	1013	89	0.73	100	0.59	100	0.75
<i>Schizophyllum commune</i> ..	812	76 *	0.79 *	85	0.88		
<i>Stereum purpureum</i>	1014	55	0.86	76	0.98	83	0.94
Brown rots:							
<i>Daedalea quercina</i>	510	3	0.50	0	0.14	2	0.41
<i>Fomes pinicola</i>	511	10	0.60	16	0.73	13	0.53
<i>Lenzites trabea</i>	1009	19	0.94	19	1.03	22	1.00
<i>Polyporus sulfureus</i>	509	0	0.05	0	0.00	9	0.43
<i>Poria monticola</i>	1010	10	0.05	0	0.05	12	0.94
<i>Ptychoqaster rubescens</i> ..	1011	4	0.02	0	0.00	2	0.47

Basal Medium: 1 % yeast extract 10 ml, 10 % NH_4NO_3 10 ml, 10 % MgSO_4 3 ml, M/l KH_2PO_4 (pH 4.5) 10 ml, distilled H_2O to 1000 ml. pH=5.1. N/l NaOH added to bring pH to 6.4. Incubation time: 4 weeks at 30° C, except where * is used to indicate a 2 week incubation. 10 replicates per trial.

T. S.=Tensile Strength; Cx activity measured in mg/ml/hr.

idase. Wood rotting fungi have long been classified as white rot — or brown rot — fungi, the former attacking both cellulose and lignin; the latter only cellulose. Our data suggest a further difference between the two types, eg. that cotton duck is much more resistant to attack by brown rot than by white rot fungi. Both types apparently exert some action on the cotton, but loss in tensile strength is much greater as a result of growth of the white rotters. Dr. Paul Marsh (1951) has reached the same conclusion from experiments of a similar nature. The brown rot organisms are thus similar to some *Aspergilli* and *Penicillia* which have been shown to produce Cx when grown on duck without appreciably affecting the tensile strength (Table 1 c).

These data are presented without any attempt at interpretation, with the hope that others will be able to integrate the facts. It may be that the difference hinges on the means of combination of cellulose with lignin, (or of cellulose with pectin) as affecting attackability.

The most frequently used cultural difference between white and brown rot organisms is the ability of the former to oxidize (extracellularly) tannic or gallic acid. White rot fungi possess polyphenol oxidase and have the ability to bring about loss in tensile strength; brown rot fungi lack polyphenol oxidase and can not bring about loss in tensile strength. The question arises »Is the ability to reduce the tensile strength of cotton always correlated with this presence of polyphenol oxidase?«.

Filtrates of wood decay fungi grown on cotton duck strips were tested for polyphenol oxidase. All that gave a positive reaction were white rotters. The filtrate of *Pholiota adiposa* was particularly active. All brown rotters and *Schizophyllum commune* gave a negative test. The latter organism, as shown by Davidson *et al.* (1938), is a borderline case giving a positive reaction with tannic but a negative reaction with gallic acid. If polyphenol oxidase is responsible for all tensile strength losses, then filtrates of other organisms actively degrading cotton duck should contain this enzyme. Unfortunately for this hypothesis, examination of filtrates of a great many of our most active cellulolytic organisms has failed to detect this enzyme. Exceptions, i.e. organisms producing a polyphenol oxidase readily when grown on cotton duck in test tubes, are *Pestalotia palmarum* QM 381, *Periconia sp.* QM 1036, *Chaetomium succineum* QM 1044, *Dictyosporium opacum* QM 1047. The oxidase is produced also when *Pestalotia palmarum* is grown on wool. Its appearance, therefore, is not in response to a substance peculiar to cotton. In shake flask tests, the enzyme appears readily when cotton duck is used as carbon source, but not when wood cellulose is used under the same conditions. We must conclude, then, that while certain cellulolytic fungi grown on duck produce a polyphenol oxidase, the presence of this enzyme extracellularly is so infrequent among fungi that its role in degradation of cotton cellulose is most unlikely.

V. *Pectinase Production by Cellulolytic Fungi.* A measurement which we have used in considering the mechanism of cellulose breakdown by cell-free filtrates is the ratio of the rate of breakdown of cotton sliver to the rate of hydrolysis (Cx) of carboxymethyl cellulose. We have found that *Trichoderma viride* filtrates attack native cotton much more rapidly per Cx unit than do filtrates of *Myrothecium verrucaria*, or of *Aspergillus niger* QM 877. It was also noted that the *T. viride* filtrates contained some pectinase while the filtrates of the other two organisms lacked pectinase (Table 6). Is the pectinase contained in the *T. viride* filtrate the basis for the greater relative activity of this filtrate on cotton sliver?

If pectinase plays an essential role, then cellulolytic organisms grown on cotton should produce pectinase — as they do Cx — and this enzyme should

Table 6. *Correlation of Cotton Activity per Cx Unit with Pectinase Activity in Filtrates.*

Source of Filtrate	QM No.	Ratio ¹ $\frac{\text{Sliver activity}}{\text{Cx activity}}$	Pectinase activity mg/ml/hr
<i>Trichoderma viride</i>	6 a	1.3	0.38
<i>Aspergillus fumigatus</i>	45 h	0.9	0.35
<i>Actinomyces sp.</i>	B 814	0.7	0.02
<i>Myrothecium verrucaria</i>	460	0.3	0.00
<i>Aspergillus flavus</i>	10 e	0.35	0.05
<i>Aspergillus niger</i>	877	0.2	0.00
<i>Penicillium pusillum</i> Smith	137 g	0.15	0.01

¹ Activity: Based on 16 hrs. activity on cotton per Cx unit.

be detectable in the outer solution. Our tests reveal that relatively few of the filtrates of the cellulolytic fungi contain pectinase.

High pectinase concentration in medium: *Aspergillus fumigatus*, *A. fischeri*, *A. luchuensis*, *Botrytis cinerea*, *Penicillium chrysogenum*, *P. citrinum*, *P. jenseni*, *Trichoderma viride* QM 6 a.

Pectinase not detectable: *Actinomyces sp.*, *Aspergillus niger* QM 877, *A. phoenicis*, *Chaetomium globosum*, *Ch. elatum*, *Fusarium sp.* QM 38 g, *Myrothecium verrucaria*, *Penicillium pusillum* Smith, *Pestalotia palmarum*, *Scopulariopsis brevicaulis*, *Torula sp.* QM 986, *Trichoderma viride* QM 13 b.

Many of these organisms will grow on pectic acid medium, and are thus able to hydrolyze the pectic acid. But of eight organisms grown on this carbohydrate, only two, *Aspergillus fumigatus*, and *Penicillium pusillum* Smith, showed an appreciable amount of the enzyme, pectinase, in the medium. Both filtrates had negligible Cx values. In an attempt to determine whether pectinase played a role in the degradation of cotton, ground, dewaxed cotton fibers were incubated with these two filtrates for 16 hours, prior to hydrolysis by Cx-containing filtrates lacking in pectinase. If pectinase were C₁, the subsequent action of the later (Cx containing) filtrates should be much greater on the de-pectinized than on the untreated fiber. Actually the hydrolysis rates both of enzyme de-pectinized and of untreated fibers were essentially the same. The data seem to support the view that pectinase is not a factor contributing to the enzymatic breakdown of the cotton fiber.

Discussion

Any positive conclusions which can arise from a discussion of the mechanism of cellulose breakdown by microorganisms are perforce limited by uncertainties as to the actual molecular structure of cellulose on the one hand and by insufficient data regarding the enzymes involved on the other. The data recorded here are intended to supplement those reported earlier, and are compatible with the mechanism previously postulated.

Some organisms (*Aspergillus flavus*, etc.) have little or no effect on the tensile strength of duck, yet produce appreciable amounts of Cx when grown thereon. Since it has been shown that these organisms produce Cx only when grown on substrata containing the 1,4 β -glucosidic linkages in long chains, it must be concluded that the organism is growing on some fraction of the cellulose. We consider such cases to be important, in that it appears that these fungi are able to attack only a small part of the cellulose of cotton — the small amount being amorphous cellulose if one is guided by the current crystalline-amorphous ideas as to the structure of cellulose. If this reasoning is followed, we must conclude that removal of this amorphous cellulose has little effect on tensile strength. It is here that we run up against a large blank in our knowledge, i.e. what factors determine the tensile strength of the cotton fiber.

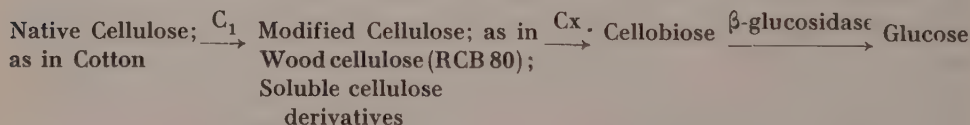
Going from the special case of *A. flavus* to that of the highly cellulolytic *Myrothecium verrucaria*, we are confronted with the apparently contradictory data of Blum and Stahl (1952) who show that the first detectable effect of filtrates of the latter organism on cotton fiber is to decrease the tensile strength. There are no early significant changes in weight, degree of polymerization, degree of crystallinity, or reducing sugars. Actually we believe all these data to be consistent. *M. verrucaria* is an active cellulolytic fungus (not in the class with those special organisms discussed above). Growing on duck, it rapidly lowers the tensile strength, apparently using both crystalline and amorphous cellulose. We postulate that active cellulolytic fungi, like *M. verrucaria*, possess factor(s) (C_1) capable of preparing the native cellulose for action by Cx. *Aspergillus flavus* and similar organisms either lack C_1 or have only an amount of very low magnitude. The data of Blum and Stahl and our own become consistent if the loss in tensile strength is attributed to C_1 . Again we must admit that this tells nothing about how C_1 brings about such a loss. In our search for an explanation, the possible action of non-cellulolytic enzymes in the filtrates was investigated. While pectinase and polyphenol oxidase have been found in many filtrates, the enzymatic hydrolysis of native cotton was independent of either. Siu (1952) boldly postulates a hydrogen-bondase equivalent to our C_1 which would aid in

separation of the chains in a crystalline area, these chains then to be available to Cx. We would prefer not to postulate a specific enzyme for which there are no known analogous types. We believe that when more is known about the structure of cellulose, the action of C₁ will become clear.

Another bit of data that we consider important is the availability of the cellulose of the cellulose-pentosan mixture (RCB 80) to those organisms (*A. flavus*, etc.) unable to produce loss in tensile strength of cotton. The obvious answer again is that here we have a higher per cent of amorphous cellulose. We have no data as to the per cent crystallinity of these materials. Several explanations of the data may be offered:

- (a) That the presence of pentosan permits *A. flavus* to get started and that the organism can then swing over to utilization of cellulose. The likelihood of this being so is minimized by the observed fact that glucose added to a cotton medium does not enable the organism to attack the cotton cellulose.
- (b) That *A. flavus* and similar organisms can use amorphous but not crystalline cellulose. This is the explanation considered above in conformance with the current fad. This possibility is weakened by two further observations (1) other wood cellulose (2 per cent pentosan) is resistant to *A. flavus*, (2) treatment of linter cellulose with 35 per cent NaOH does not make it available to *A. flavus*.
- (c) That the chemical changes occurring during preparation of wood cellulose (RCB 80) are of the same nature as that brought about by C₁. Then organisms deficient in C₁ are able to attack the modified cellulose.

On the basis of the data presented in this paper, we can supplement the scheme previously proposed by the insertion of specific organisms as follows:



I. Organisms producing C₁, C_x and β -glucosidase

M. verrucaria, *A. terreus*, *A. fumigatus*, *A. niger* QM 877

II. Organisms producing C_x and β -glucosidase

A. flavus, *A. sydowi*, *A. tamarii*, *A. niger* QM 458

III. Organisms producing β -glucosidase

Most fungi; Phycomycetes slow

It is apparent that we are translating data obtained using filtrates to the action of the organism on the same substratum. The very purpose of a study of filtrate activity is to find out what is taking place outside of the fungus. But we should not lose sight of the differences involved: (1) The fungus absorbs the products of extracellular activity, while in enzyme hydrolysates the end products accumulate. (2) Filtrates are active over the entire substrate surface. The organism is active locally, the activity being concentrated chiefly at the hyphal tip, and decreasing with distance from the tip. Such differences in localization of activity may explain differences in results where the measurements made are of D.P., degree of crystallinity, and tensile strength.

We have used yet another test for our hypothesis. If the concept of a C_1 step is valid, then filtrates from weakly cellulolytic organisms should have low activity on cotton because they lack, or have very little of, the initiating enzyme C_1 . This is generally true. *A. flavus* and *A. sydowi*, for example, when grown on wood cellulose produce filtrates which contain 1 to 2 Cx μ /ml, and produce 0.0—0.3 mg glucose/ml from cotton in 16 hours. Filtrates of active cellulolytic organisms like *A. fumigatus* and *Trichoderma viride* having the same Cx unitage produce 0.6—1.0+mg glucose. In our experiments we have found as high as 40-fold differences between filtrates of equal Cx activity (in Table 6, the range is 9-fold) in their ability to hydrolyze cotton cellulose. We believe that such differences are due to the amount of C_1 present. At the same time, we readily admit that we have no way of knowing just how closely our determination of Cx activity agrees with Cx activity on unsubstituted cellulose. The best we can say is that this type of data is consistent with the interpretation we make.

Recently, the pectic enzymes of several organisms have been studied. While the usual action of fungal filtrates is hydrolysis of pectic acid to galacturonic acid, the filtrates of some organisms (*Saccharomyces fragilis* (Luh and Phaff 1951), *Byssoschlamys fulva* (Beaven and Brown 1949), *Neurospora crassa*) can accomplish only a partial splitting of the substratum. Two steps are postulated (Luh *et al.* 1951), the first »disaggregating» or »depolymerase», the second polygalacturonase. Here, then, is a situation analogous to that in cellulose breakdown in which the broad approach (comparative physiology) was similar to ours, and from which the same type of mechanism was deduced.

Summary

A large number of microorganisms has been examined with respect to cellulose hydrolysis. All those which reduce tensile strength of cotton duck produce the hydrolytic enzyme, Cx. Some microorganisms which do not

reduce the tensile strength of duck also produce Cx. A scheme for hydrolysis based on attack of cellulose and cellulose derivatives by microorganisms is presented. It has been shown that neither pectinase nor polyphenol oxidase is involved in the primary breakdown of cotton cellulose.

The authors owe their thanks for critical comments and suggestions during the course of this work to their coworkers: R. G. H. Siu, G. R. Mandels, W. Stahl, R. Blum. Dr. Paul Marsh of the USDA supplied data of a similar nature to that in Table 5, and L. A. Brinkerhoff (USDA) sent us the culture of *Rhizopus* sp. (QM 1032) which he isolated from cottonseeds. Dr. Kenneth Raper of the NRRL has identified *Penicillium pusillum* Smith for us.

References

- Beaven, G. H. & Brown, F.: The Pectic Enzymes of the Mould *Byssoschlamys fulva*. — *Biochem. J.* 45: 221. 1949.
- Bhargava, K. S.: Physiological studies of some members of the family Saprolegniaceae. I. Enzyme Action. — *J. Indian Bot. Soc.* 22: 85. 1943.
- Davidson, R. W., Campbell, W. A. & Blaisdell, D. J.: Differentiation of wood-decaying fungi by their reactions on gallic or tannic acid medium. — *J. Agr. Res.* 57: 683. 1938.
- Huseman, E. & Lötterle, R.: Über den fermentativen Abbau von Polysacchariden. I. Der heterogene Abbau von Cellulose. — *Makromol. Chem.* 4: 278. 1950.
- Levinson, H. S., Mandels, G. R. & Reese, E. T.: Products of Enzymatic Hydrolysis of Cellulose. — *Arch. Biochem. and Biophys.* 31: 351. 1951.
- Levinson, H. S. & Reese, E. T.: Enzymatic hydrolysis of soluble cellulose derivatives as measured by changes in viscosity. — *J. Gen. Physiol.* 33: 601. 1950.
- Luh, B. S. & Phaff, H. J.: Studies on Polygalacturonase of Certain Yeasts. — *Arch. Biochem. and Biophys.* 33: 212. 1951.
- Marsh, P. B.: Personal communication. 1951.
- Marsh, P. B., Bollenbacker, K., Butler, M. L. & Raper, K. B.: Fungi concerned in fiber deterioration. — *Tex. Res. J.* 19: 462. 1949.
- Mehrotra, B. S.: Physiological studies of the genus *Phytophthora*. I. Enzyme Action. — *J. Indian Bot. Soc.* 28: 108. 1949.
- Norkrans, Birgitta: Studies in Growth and Cellulolytic Enzymes of *Tricholoma*. — *Symbolae Botanicae Upsaliensis* II (7): 1. 1950.
- Phaff, H. J.: The Production of Exocellular Pectic Enzymes by *Penicillium chrysogenum*. I. On the Formation and Adaptive Nature of Polygalacturonase and Pectinesterase. — *Arch. Biochem.* 13: 67. 1947.
- Ploetz, T.: Über einige Enzyme des Hausschwamms (*Merulius lacrimans*). 2. Mitt. über den enzymatischen Abbau polymerer Kohlenhydrate. — *Zeitschr. physiol. Chemie* 261: 183. 1939.
- Ray, W. W.: Cotton Boll Rots in Oklahoma. — *Okla. Bul. B* 300. 1946.
- Reese, E. T. & Downing, M. H.: Activity of the Aspergilli on Cellulose, Cellulose derivatives and wool. — *Mycologia* 43: 16. 1951.
- Reese, E. T., Siu, R. G. H., & Levinson, H. S.: Biological degradation of soluble cellulose derivatives. — *J. Bact.* 59: 485. 1950.
- Siu, R. G. H.: Microbial Decomposition of Cellulose. — Reinhold Publ. Corp. N.Y. 1951.
- In revised edition of Cellulose by E. Ott. — Interscience Publ. N.Y. 1952.
- Stahl, W. & Blum, R.: Enzymic degradation of cellulose fiber. — *Tex. Res. J.* 22: 178.
- Tracey, M. V.: Cellulase from leaves and roots of tobacco. — *Biochem. J.* 47: 431. 1950.

Studies of the Decomposition of Cellular Lipids of Molds

By

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The cellular lipids of molds may be considered in the main to constitute a nutritional reserve of the cells, which is stored up by continuing growth and with excess of suitable carbohydrate in the environment. It is known that this lipid reserve disappears by inanition. A part of the cellular lipids may be connected with other functions, since about 1.3—1.4 per cent of the dry weight of mold mycelium constitutes lipids which are not decomposed by continued inanition. This part of the cellular lipids has generally been termed »constant element of lipids»; whereas the decomposable part of the lipids is termed »variable element of lipids» (cf. Foster, 1).

Methods

A *Penicillium* strain of the monoverticillata group, not definitely identified, was employed as the test organism. Cultivation was carried out in mold flasks of Roux type on a medium composed according to Turpeinen (5): KH_2PO_4 1 g., MgSO_4 0.5 g., NaCl 0.2 g., CaCl_2 0.2 g., peptone (Difco) 3 g., and glucose 5 to 20 g. in 1000 ml tap water. Inoculations were carried out by means of spore suspensions from 3 day's slant cultures on wort agar.

Inanition experiments were carried out by the replacement technique. After sufficient mycelium formation (72 to 120 hours) the initial medium was decanted off, the mycelial mat washed several times with sterile distilled water, and a new substrate or solution poured into the flask under the mycelium.

The dry weight of the mycelium was determined by separating the mycelium by filtration and drying it at 50°C to constant weight. The continuous mat formation made it possible to obtain sufficiently accurate values for a reliable comparison of different amounts of mycelium in this way.

The lipids were extracted from ground dry mycelium by means of ether after a pretreatment with 5 N HCl, and further treatment with petroleum ether according to the micromethod described by Turpeinen (5). The lipids were determined iodometrically according to Kimmelstiel and Becker (3).

The total nitrogen of the dry mycelium was determined by ordinary Kjeldahl digestion and distillation followed by iodometric titration.

The experiments were carried out over a period of 4 days, with analyses after 2 and 4 days.

Experiments and Discussion

The test organism employed was selected for investigation quite arbitrarily in order to carry out the experiments with an organism generally representative of a greater group of common molds. It was found to produce maximally 9 to 11 per cent lipids of dry matter.

Comparison of the extent of lipid decomposition in mycelium left in its initial medium with that of similarly produced mycelium transferred to distilled water, revealed remarkable differences. In the former case the lipid content reached a minimum value of 1.1—1.2 per cent of dry matter within 4 days from the disappearance of the glucose, while in the latter case the mycelium still contained 3.6 per cent lipids after 30 day's inanition. Between the 14th and 30th day the lipid content fell only 0.2 per cent, from 3.8 to 3.6 per cent. It was further established that the loss in dry matter in the former case was only 14 per cent, while in the latter case, or in experiments carried out analogically, it could be 30 to 40 per cent in 4 days. These observations led to investigations of the relationship between the decomposition of nitrogenous compounds and that of the lipids.

Figure 1 illustrates one such experiment. This graph shows that when the lipids are decomposed to an even rate during the whole experiment, the decomposition of nitrogenous compounds simultaneously proceeds slowly, and ultimately stops. This was the case if the mycelium was not removed from its original medium (the time of complete consumption of glucose was taken as the beginning of the experiment, which explains the deviating starting point for the lipid curve 1 in Figure 1). When the mycelium is transferred to new substrates the rate of lipid decomposition decreases by continued inanition, while the rate at which the nitrogenous compounds are broken down keeps its initial strength. It seems evident that at the first interval, after the glucose was totally consumed, or the mycelium transferred to new substrates, the lipids are decomposed almost to an equal extent in every case. During the second interval, however, there is a clear inverse relationship between the decomposition of nitrogenous compounds and the decomposition of lipids.

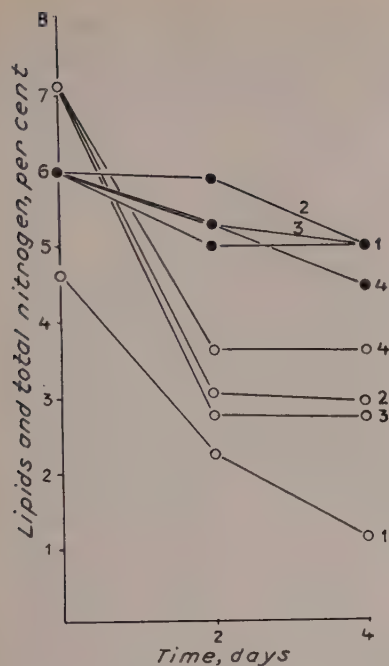


Fig. 1.

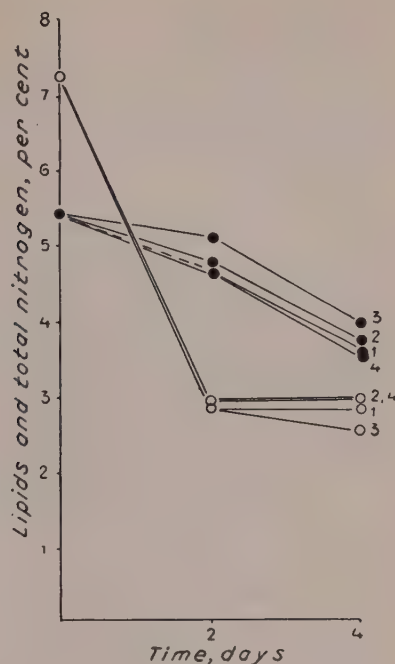


Fig. 2.

Figure 1. Changes in the contents of lipids and nitrogen of mold mycelium when transferred to different media. ○ Lipids, ● Total nitrogen; 1. In initial medium, 2. Transferred to the same medium without glucose, 3. Transferred to the same medium with 0.1 per cent glucose, 4. Transferred to distilled water.

Figure 2. Changes in the contents of lipids and nitrogen of mold mycelium when transferred to different nitrogen containing solutions. ○ Lipids, ● Total nitrogen; 1. NH_4NO_3 , 2. Urea, 3. Glycine, 4. Peptone.

It may be mentioned further that (as can also be seen from the Figure 1) the nitrogen present in new substrates does not influence the breakdown of cellular nitrogenous compounds, which seems to be decisive for the lipid decomposition. This was also found in an experiment where the mycelium was transferred to solutions containing ammonium nitrate, urea, glycine or peptone, respectively. In these solutions the lipids of the mycelium were decomposed in a similar way to that described above (Figure 1; curves 2 and 3), rapidly immediately after the transfer and then the ceasing of decomposition, whereas the nitrogenous compounds were broken down to an increasing extent (Figure 2).

No attempts have been made in the present investigation to explain the «sparing effect» of undisturbed cultural conditions on the nitrogenous cell

constitutents, but it seems evident that the lipid decomposition ceases if a rapid decomposition of nitrogenous compounds takes place, and, conversely, if the nitrogenous compounds are only slightly decomposed the degradation of lipids continues until the lipid reserve, the »variable element» of lipids is completely broken down.

When the »variable element» was not totally decomposed a lipid content of 2.8—3.6 per cent of dry matter could still be found. This final lipid content generally was reached within the first test interval (2 days).

The idea of enzymatic adaption, first presented by Karström (2) as regards bacterial enzymes, has been found to hold good for the fungi also, although less attention has been paid to this phenomenon with these organisms (cf. Foster 1). During recent years Virtanen and his co-workers (6—9), in their investigations into bacterial and yeast enzymes, have established that in cells grown on media poor in nitrogen the protein components of some enzymes are synthesized in relatively invariable amounts, whereas the amount of other enzymes decreases rapidly with the lowered nitrogen content of the cells. Because of this fact enzymes of the former type have been termed indispensable and those of the latter type dispensable enzymes.

If Virtanen's finding is applied to the degradation of proteins in old cells by inanition, the supposition may hold good that the cell proteins involved in dispensable enzymatic processes are primarily decomposed or transformed into indispensable enzyme proteins. If this is correct, the inanition may lead to a successive decrease in the rate of such enzymatic processes that are dispensable in the metabolism of the cell during inanition.

When the discussion outlined above is applied to the topical problem, the question arises as to whether the lipid decomposition may be considered indispensable or not. It follows, from the nature of the storing up of cellular lipids, that the enzymes active in this case may be dispensable components of the cell, but may nevertheless be present when lipids have been synthesized. In fact, the enzyme system involved in lipid synthesis may be responsible for the reverse process — the lipid decomposition. During inanition of mold cultures a rapid loss in amounts of total cell substance (dry mycelium) occurs under certain conditions. This degradation, which is not checked by the decrease in lipid amounts, must of course affect other components of the cell also, among them the proteins. Thus the tendency of the cells to liberate the required energy by decomposition of lipids during the first stages of inanition is gradually suppressed when the decomposition of proteins reaches a point where the lipolytic activity decreases. Ultimately lipid decomposition ceases and proteins remain as the sole source of energy, which further accelerates the decomposition of the proteins.

The literature contains no mention of the influence of the nitrogen metabolism of the mold mycelium on lipid formation. However, as noted by Starkey (4), and a number of earlier investigators, a low nitrogen content of the medium promotes lipid formation of some groups of microorganisms.

Summary

The decomposition of the mycelial reserve lipids of molds has been investigated. Employing a *Penicillium* strain as the test organism it was found that the extent of lipid decomposition in the mycelium during inanition depends on the simultaneous decomposition of nitrogenous cell components, in that an extensive loss of cell nitrogen leads to a remarkable decrease in lipid decomposition, especially when the inanition is continued over periods longer than two days.

References

1. Foster, J. W.: Chemical Activities of Fungi. — Academic Press, New York, pp. 114—137. 1949.
2. Karström, H.: Über die Enzyymbildung in Bakterien und über einige physiologische Eigenschaften der untersuchten Bakterien. — Ann. Acad. Sci. Fen., A, 33: 2. 1930.
3. Kimmelstiel, P. & Becker, H.: Die titrimetrische Fettbestimmung in kleinen Organismen. — Z. Physiol. Chem. 209: 166. 1932.
4. Starkey, R. L.: Lipid Production by a Soil Yeast. — J. Bact. 51: 33. 1946.
5. Turpeinen, O.: Studies on Mold Lipids. — Ann. Acad. Sci. Fen., A, 46: 4. 1936.
6. Virtanen, A. I.: On the Adaptive Formation of Enzymes by Microorganisms. — Sv. Kem. Tidskrift 60: 23. 1948.
7. — Dependence of the Enzyme Activity of Cells on their Protein Content. — Ann. Acad. Sci. Fen., A 2: 36. 1949.
8. — & de Ley, J.: The Enzyme Activity and Nitrogen Content of Bacterial Cells. — Arch. Biochem. 16: 169. 1948.
9. — & Winkler, U.: Effect of Decrease in the Protein Content of Cells on the Proteolytic Enzyme System. — Acta. Chem. Scand. 3: 272. 1949.

Inorganic Carbon Sources of Green Algae. V. Inhibition of Photosynthesis by Cyanide

By

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Introduction

The experiments published in this series of papers have been discussed on the assumption that *Scenedesmus quadricauda* can use both CO_2 and HCO_3^- . According to Rabinowitch (1951, p. 891 and 908), the results may also be explained if only CO_2 is used in photosynthesis. In such a case, however, some rather improbable assumptions must be made. Photosynthesis is very rapid even in alkaline solutions at low total concentration of carbonic acid. Confer, for example, Österlind (1951 b, Fig. 1) where a very high rate is reached at pH 9.2 at a total concentration of 100 μM carbonic acid, corresponding to a CO_2 concentration of only 0.06 μM . Such a low concentration may not be used at all at low pH values (Österlind 1951 a, Fig. 7), and according to Rabinowitch (1951, Table 27. I), the lowest value ever reached for full saturation of photosynthesis in algae is 5 μM . But the conditions for using CO_2 may be quite different at high and at low pH values.

In cultures of *Scenedesmus quadricauda*, aerated with air (0.03 per cent CO_2) (Österlind 1949, Fig. 31), the great difference between the growth rate at pH 5.5 and 6.5 is difficult to explain if HCO_3^- ions are not used. It has been assumed possibly to be a pH effect (Myers 1951), but according to unpublished experiments, the rate of photosynthesis at optimal concentration of the carbon sources is constant between pH 3.5 and 9. The assumption of Steemann Nielsen (1952, p. 153) that the photosynthesis of *Scenedesmus quadricauda* (not *Sc. obliquus*!) is influenced by variations in pH does not seem to be correct. However, growth determinations and photosynthesis experiments do not always agree as shown by the curve representing 6.1 per

cent CO_2 (Österlind 1949, Fig. 31), according to which the growth rate at pH 3.5 is only 50 per cent of that at pH 7. This difference between growth and photosynthesis may be due to the photometric method used in determining the density of the solutions. It seems, however, improbable that the great difference in growth between pH 5.5 and 6.5 in 0.03 per cent CO_2 could be explained in this way.

As an attempt to find differences between the mechanisms of absorption of CO_2 molecules and HCO_3^- ions, the inhibiting effect of KCN on photosynthesis was determined at various pH values. Cyanide is a suitable inhibitor in such experiments, due to the high pK_a value (cf. Österlind 1952).

Experiments

The experiments were performed by means of the previously described vessels (Österlind 1951 c). The algae were cultured during 5 or 6 days in nutrient solution C containing HCl (Österlind 1949, p. 41), aerated with ordinary air. At the harvesting of the cells, the pH was about 9.5. The cells were centrifuged and suspended in 0.1 M NaK-phosphate buffer of the desired pH. To the ampulla was added a mixture of Na_2CO_3 and KCN. The amount of Na_2CO_3 corresponded to a total concentration of 1 mM after it had been added to the phosphate buffer. At the start of the experiment, the carbon sources of the cells and the phosphate buffer were emptied by allowing the cells to photosynthesize until oxygen production had ceased. The content of the ampulla was then added in the dark. After 10–15 minutes the vessels were again illuminated and the reading of the manometers begun. The experiments were performed at various pH values, the data obtained being shown in Figures 1 and 2, where the rate of photosynthesis is given in terms of per cent of control without KCN. The concentrations of CO_2 and HCO_3^- are also noted.

Discussion

At pH 5.8 and 6.0, the inhibiting effect of KCN is low, 10 μM causing an insignificant inhibition and 50 μM causing about 30 per cent inhibition. With increasing pH values, the sensitivity of photosynthesis to cyanide increases, the highest sensitivity being reached a little above pH 7, remaining almost constant between this point and pH 8.5. At even higher pH values the sensitivity decreases. Undoubtedly, this depends on the low concentration of active HCN, part of the added cyanide occurring as inactive CN^- ions. The concentrations of undissociated HCN have been calculated and these values

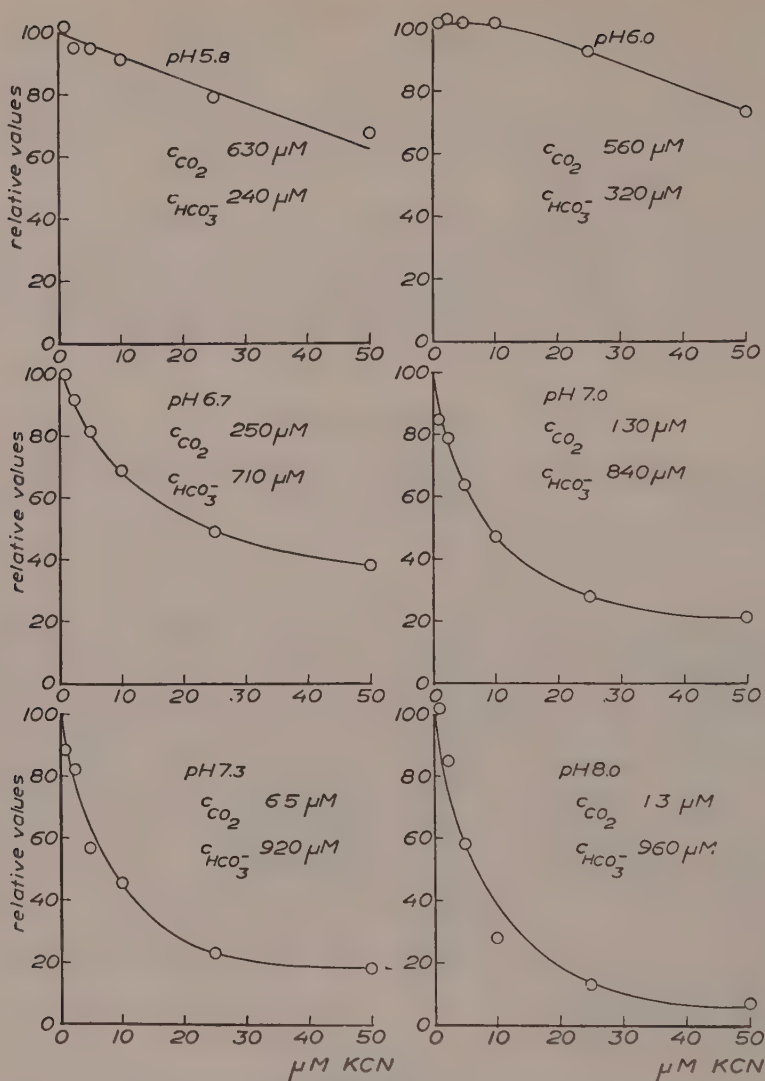


Fig. 1. Inhibition of photosynthesis of *Scenedesmus quadricauda* at various pH values and various concentrations of KCN.

have been used in the broken curves. When only the HCN concentration is taken into consideration, the sensitivity of photosynthesis is constant also at high pH values.

As was mentioned above, the inhibition caused by KCN at pH 5.8 and 6.0 is very low. Thus, CO_2 or HCO_3^- or both may be taken up and used in the

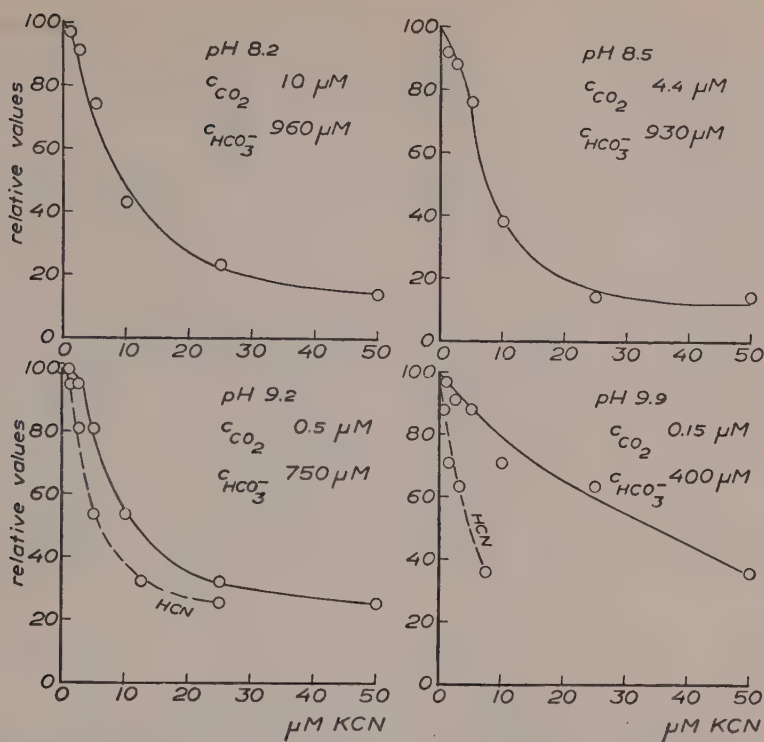


Fig. 2. Inhibition of photosynthesis of *Scenedesmus quadricauda* at various pH values and various concentrations of KCN. The broken curves refer to the concentration of undissociated HCN.

presence of KCN. At pH 6.7 and 7.0, the concentration of CO_2 and HCO_3^- are both great enough to permit maximal photosynthesis by only one of them. In spite of this, the inhibition by KCN is much greater than at pH 6.0. As the concentration of HCO_3^- is higher than at the lower pH values, this greater inhibition must be due to the lower concentration of CO_2 or to the difference in pH. The two experiments described below show that the difference in inhibition is due to the concentration of CO_2 . Thus, the inhibition of the assimilation of CO_2 by KCN is of the competitive type, being reduced by a surplus of the substrate CO_2 .

It was my intention that the experiments with cyanide inhibition would make it possible to decide 1) if only carbon dioxide molecules are used in photosynthesis also at high pH values, or 2) if also bicarbonate ions are used. It seems, however, as if the results may be explained according to both these theories.

1) At low concentration of the substrate, the sensitivity of the reaction to a certain concentration of the enzyme inhibitor does not increase markedly even with an inhibitor of the competitive type. Therefore, the experiments may be explained by assuming that only CO_2 is assimilated. At low concentrations of CO_2 (high pH), the reaction is inhibited to 40 per cent by $10 \mu\text{M}$ KCN, while at higher concentrations CO_2 competes with the cyanide to unite with the enzyme molecules, the sensitivity of the reaction to KCN thus being decreased.

2) If bicarbonate ions are also assimilated (an assumption which seems to be the correct one with respect to previous results), the experiments may be explained in the following way. At all pH values, HCO_3^- is present in great surplus but in spite of this its assimilation is very sensitive to KCN, *this inhibition of the bicarbonate assimilation being uncompetitive*. At pH 5.8 and 6.0, this inhibition does not reduce the rate of photosynthesis due to the surplus of CO_2 . At pH 6.7 and 7.0, the assimilation of CO_2 is inhibited as explained above. At higher pH values, the assimilation of CO_2 is very slow even when KCN is absent, but the HCO_3^- assimilation is inhibited.

Control Experiments

It was assumed above that the increasing sensitivity of photosynthesis to KCN at pH 6.7 and 7.0 is due either to the decreasing CO_2 concentration or to the increasing pH. Two control experiments were made to decide between these possibilities.

Table 1 (Expt. 1). *Inhibition of photosynthesis at various pH values by KCN at a CO_2 concentration of about $500 \mu\text{M}$.*

No. of vessel	pH	Na_2CO_3 added μM	C_{CO_2} μM	$\text{C}_{\text{HCO}_3^-}$ μM	C_{KCN} μM	Rate of photosynthesis	Inhibition to %
1	5.95	1000	580	300	0	55	
2	5.95	1000	580	300	10	45	82
3	6.65	2000	500	1400	0	57	
4	6.65	2000	500	1400	10	50	88
5	7.20	5000	450	4500	0	58	
6	7.20	5000	450	4500	10	50	86

In Expt. 1 (Table 1), three pH values were used. By adding various amounts of Na_2CO_3 to the vessels, almost the same CO_2 concentration (about $500 \mu\text{M}$) was obtained at all three pH values. One vessel at each pH received no KCN,

while 10 μM KCN was added to the other. The inhibition is almost the same at all pH values and the mean values (85 per cent photosynthesis in 10 μM KCN) is in a rather good agreement with the result of the main experiments.

Table 2 (Expt. 2). *Inhibition of photosynthesis at various pH values by KCN at a CO_2 concentration of about 225 μM .*

No. of vessel	pH	Na_2CO_3 added μM	C_{CO_2} μM	$\text{C}_{\text{HCO}_3^-}$ μM	C_{KCN} μM	Rate of photosynthesis	Inhibition to %
1	5.90	400	240	110	0	46	52
2	5.90	400	240	110	10	24	
3	6.60	800	225	540	0	54	50
4	6.60	800	225	540	10	27	
5	7.10	2000	220	1750	0	54	54
6	7.10	2000	220	1750	10	29	

Expt. 2 (Table 2) was made according to the same method but the CO_2 concentration was only about 225 μM at all pH values. In agreement with the main experiments, the inhibition was about 50 per cent in 10 μM KCN and independent of the variations in pH, which also in this experiment included the pH interval in the main experiments which would be examined.

Summary

The inhibition of the photosynthesis of *Scenedesmus quadricauda* by KCN at various pH values and at various concentrations of CO_2 has been studied. When the CO_2 concentration exceeds 500 μM , the inhibition by 10 μM KCN is very low. At limiting concentrations (100—200 μM), the inhibition of CO_2 assimilation is more pronounced. This inhibition seems to be competitive.

At high pH values, the inhibition is strong which may be explained either by assuming a continuation of the competitive inhibition of the CO_2 assimilation or as an incompetitive inhibition of HCO_3^- which is present in a great surplus.

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References

- Myers, J.: Physiology of the algae. — Ann. Rev. Microbiol. — 5: 157. 1951.
- Österlind, S.: Growth conditions of the alga *Scenedesmus quadricauda* with special reference to the inorganic carbon sources. — Symbol. Botan. Upsaliens. 10 (3): 1. 1949.
- Inorganic carbon sources of green algae. III. Measurements of photosynthesis in *Scenedesmus quadricauda* and *Chlorella pyrenoidosa*. — Physiol. Plant. 4: 242. 1951 a.
- Inorganic carbon sources of green algae. IV. Photoactivation of some factor necessary for bicarbonate assimilation. — Physiol. Plant. 4: 514. 1951 b.
- Anion absorption by an alga with cyanide resistant respiration. — Physiol. Plant. 4: 528. 1951 c.
- Rabinowitch, E. I.: Photosynthesis and related processes. Vol. II. Part 1. — New York. 1951.
- Steemann Nielsen, E.: Experimental carbon dioxide curves in photosynthesis. — Physiol. Plant. 5: 145. 1952.

Effect of Cellobiose on the Enzymatic Hydrolysis of Cellulose and its Derivatives

By

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In a previous study of the products of cellulose hydrolysis by microbial filtrates, cellobiose was found to be the end-product of the reaction mediated by the 1,4 β -polyglucosidase, Cx (Levinson *et al.* 1951). In actively growing cultures the further metabolism of cellobiose occurs *within* the organism. It has been shown that the cleavage products of enzymic hydrolysis of carbohydrates often are inhibitors of the enzyme concerned, e.g. glucose and fructose inhibit the activity of invertase, maltose that of amylase. If cellobiose is indeed the product of Cx activity, it might be possible to inhibit the reaction by the addition of cellobiose. Karrer *et al.* (1924) have shown that cellobiose (0.075 per cent) inhibits lichenin hydrolysis by snail lichenase, but glucose, fructose and galactose had an almost equal effect. Norkrans (1950) has shown that cellobiose (0.03 per cent) inhibits hydrolysis of cellulose (reprecipitated from cuprammonium solution) by extracellular, cellulolytic enzymes of the fungus, *Tricholoma nudum*. No other sugars were used for comparison.

This paper is restricted to a consideration of the effect of cellobiose on the hydrolysis of several cellulosic substrata by cell-free filtrates of various microorganisms. Other sugars and methyl cellulose have been used to determine whether the effects observed are peculiar to cellobiose.

Methods

Three different cellulosic substrata were used: a cellulose derivative (carboxymethylcellulose), a swollen linter cellulose, a native cellulose (ground,

¹ Part of this work was done during a visit by Dr. Norkrans to our laboratory.

dewaxed cotton fiber 40—60 mesh). Most of the experiments were performed with carboxymethylcellulose (CMC) and the hydrolysis was followed in a Hoeppler viscosimeter (Levinson *et al.* 1951). The results were based on a conversion of the fall time of the ball to specific viscosity (η_{sp}):

$$\eta_{sp} = \frac{\eta_{\text{solution}}}{\eta_{\text{solvent}}} - 1$$

and $\eta_{sp} = c F - 1$ where F = fall time of ball

$$\Phi = \frac{1}{\eta_{sp}} = \frac{1}{c F - 1}$$

c is a constant that includes characteristics of ball and solution — under the test conditions

and Φ = specific fluidity

The reaction mixture contained 1 per cent of CMC in 0.05 *M* citrate buffer. Most routine measurements were made at pH 5.4—5.6, and at a temperature of 35° C. The rate of change in fluidity is a function of the enzyme activity.

The swollen cellulose was prepared by treatment of linter cellulose with 85 per cent phosphoric acid as described by Walseth (1948). The washed material remains well suspended. The extent of hydrolysis of the native and swollen cotton cellulose was measured gravimetrically. Three to four hours enzymic treatment at 50° C was sufficient to give a measurable loss in weight of the swollen cellulose. The native cellulose was incubated 18—24 hours at 40° C.

In the CMC viscosity test, very low enzyme concentrations are required (0.3—2.0 per cent, based on volume of enzyme filtrate). In the swollen cellulose tests, the filtrate represented 30 per cent, and in the cotton tests 90 per cent of the total volume of suspension. The substrate concentration varied with the tests, being one per cent for CMC and for ground cotton, and about 0.75 per cent for the swollen cellulose.

The enzymes used were prepared by growing the organism in shake flasks on a cellulosic substratum (Reese *et al.* 1950). The residue was removed by filtration, and the cell-free filtrates preserved with 0.01 per cent merthiolate in a refrigerator.

Only commercially available materials were used. Two sources of cellobiose were employed (Pfanstiehl and Difco). The CMC was obtained from Hercules Powder Company as grade 70 M, and is of medium viscosity. The methocel is a product of Dow Chemical Company and has a degree of substitution (DS) of about 1.8.

Results

(1) Hydrolysis of CMC (viscosity data)

We first observed cellobiose inhibition in our laboratory by the viscosity method. Filtrates of cultures of several organisms were tested (Table 1), and all but two showed inhibition of Cx activity by 2 per cent cellobiose. The two exceptions, filtrates of *Streptomyces sp.* and of *Myrothecium verrucaria*, were

Table 1. *Effect of cellobiose and of glucose on hydrolysis of CMC by the Cx's of various organisms (Viscosimetric Method).*

Filtrate of	QM No.	Cellobiose		Glucose	
		pH 5.0	7.0	5.0	7.0
<i>Aspergillus flavus</i>	10 e	I	I	0	
<i>Aspergillus fumigatus</i>	45 h	I			
<i>Aspergillus niger</i>	458	I			
<i>Memnoniella echinata</i>	1 c	I			
<i>Myrothecium verrucaria</i>	460	S	0		
<i>Penicillium pusillum</i>	137 g	I	I	0	0
<i>Pestalotia palmarum</i>	381	I		0	
<i>Streptomyces</i> sp.	B 814	S	I	0	0
<i>Trichoderma viride</i>	6 a	I			
<i>Torula</i> sp.	986	I		S	

I = Inhibition; S = Stimulation; 0 = No effect.

stimulated by the addition of cellobiose. It was this peculiar behavior that led us to look further into the problem.

Greatest stimulation was observed in the case of the *Streptomyces* sp. filtrate. To determine whether stimulation was general among members of this genus, five other cellulolytic isolates were tested. Three of these were stimulated by 1 per cent cellobiose (95—169 per cent stimulation); and two were inhibited (33—62 per cent inhibition). The isolate most studied (QM B 814) produced a filtrate stimulated under the same conditions to an extent of 260 per cent.

In all, filtrates of 36 organisms were tested for the effect of 2 per cent cellobiose on the hydrolysis of CMC at pH 5.4. These include representatives of all major groups of fungi, several actinomycetes and one bacterium (*Sporocytophaga myxococcoides*). Of these 26 were inhibited, 3 were unaffected, and 7 were stimulated. Glucose had a stimulatory effect in 2 cases where cellobiose inhibited (*Trichoderma viride*, *Torula* sp.); in all other cases glucose was without effect. Those filtrates that were stimulated by cellobiose include the 4 strains of *Streptomyces* (above), *Myrothecium verrucaria*, *Chaetomium elatum* QM 606, and *Fusarium roseum* QM 38 g. The range of activity covered by cellobiose is from over 60 per cent inhibition (*Streptomyces* sp. JTB 2604 and unidentified Basidiomycete cultures) to over 200 per cent stimulation (*Streptomyces* sp. QM B 814).

For further tests, two organisms (*Streptomyces* sp. QM B 814; *Myrothecium verrucaria* QM 460) were selected to represent those whose filtrates were stimulated by cellobiose, and two organisms (*Trichoderma viride* QM 6 a; *Penicillium pusillum* QM 137 g) to represent those whose filtrates were inhibited. Increasing the cellobiose concentration (Figure 3) in the range

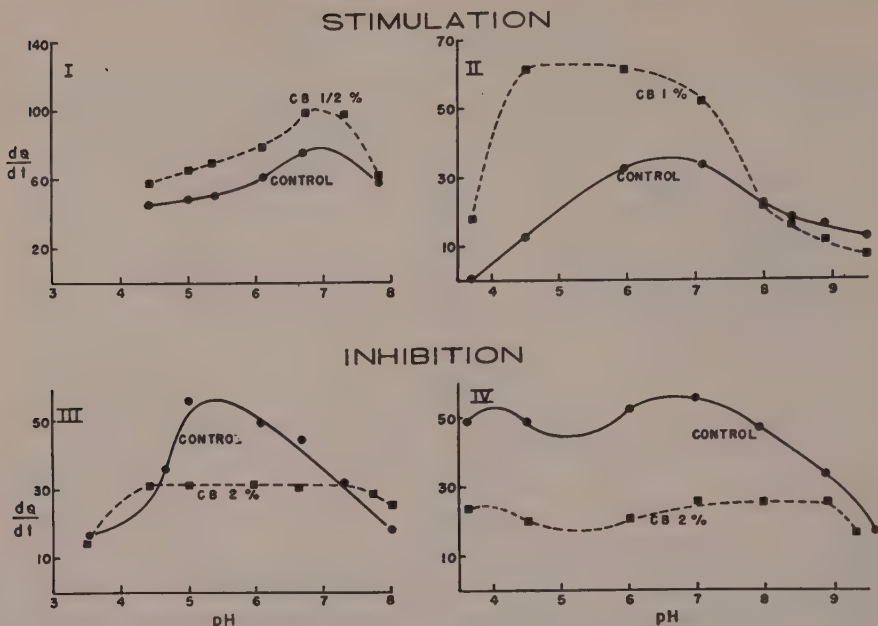


Figure 1. Effect of pH on stimulation and inhibition by cellobiose as measured by changes in fluidity (CMC).

- I *Myrothecium verrucaria* - - - - with cellobiose
 II *Streptomyces sp.* ——— without cellobiose
 III *Trichoderma viride*
 IV *Penicillium pusillum*

$\frac{d\sigma}{dt}$ is the rate of change of fluidity; $\sigma = \frac{1}{\eta_{sp}}$

1—4 per cent, increased the inhibition. Where stimulation occurred, the optimum concentration varied with the source being 1 per cent for *Streptomyces sp.* and 0.5 per cent for *Myrothecium verrucaria* under the conditions of this test.

Hydrogen ion concentration has a marked effect on the amount of inhibition or of stimulation produced by addition of cellobiose. These effects on the shape of the pH-activity curves are shown in Figure 1. For *Streptomyces sp.* filtrate, the addition of cellobiose increases the rate of hydrolysis and broadens the zone of optimum activity. As a result, at pH 4.5 where the control activity is about $\frac{1}{3}$ maximal, the test solution containing cellobiose remains at its maximum value. Such differences in the shapes of the curves lead to different amounts of stimulation or of inhibition at the various pH levels.

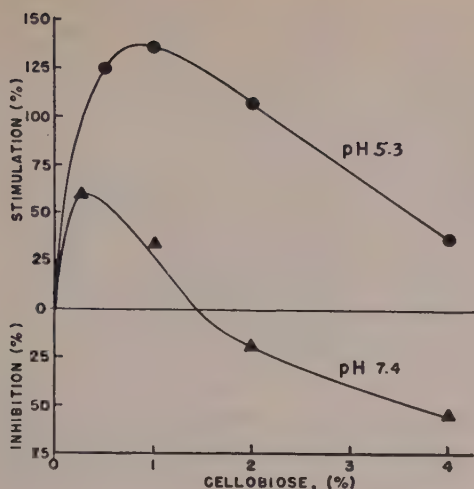


Figure 2. Effect of cellobiose concentration on stimulation of *Streptomyces* sp. filtrate at various pH's (CMC).

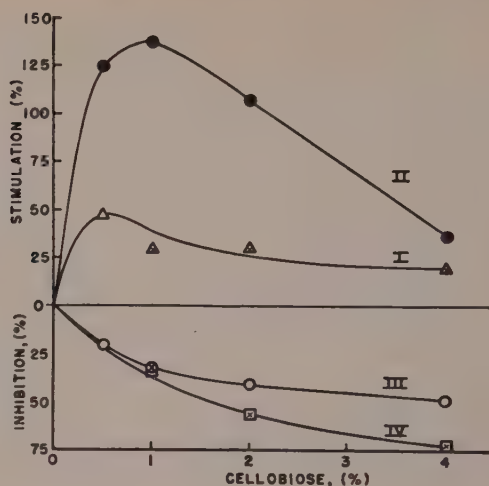
A flattening out of the pH-activity curve by cellobiose similar to that observed above is found with *Trichoderma viride* filtrates. In this case, however, the effect of the cellobiose is to inhibit. For the other two filtrates the control and cellobiose curves remain more or less parallel over most of the range. It is obvious that we have drawn the pH-activity curve for *Penicillium pusillum* with little regard for the experimental points. The curves have been so drawn on the basis of additional data showing a bimodal nature. A similar curve for pH vs cellulase activity has been reported for the enzymes of the larvae of *Stromatium fulvum* (Mansour and Mansour-Bek 1937). Such bimodal pH-activity curves appear to be rare. We suppose they may indicate the presence of two cellulases of different pH optima. Except for *Streptomyces* sp., the region of maximum cellobiose effect appears to be near the pH range of optimal activity. Most interesting is the decrease in stimulation (and in inhibition) as the pH rises, and the change from stimulation to inhibition (and vice versa).

There is an interaction between cellobiose concentration and pH. This was suggested by a comparison of some of the earlier data with later results. In Table 1, it is indicated that for *Streptomyces* sp. B 814, stimulation was observed at pH 5.0 and inhibition at pH 7.0. This is true for all cellobiose concentrations above 1.5 per cent (Figure 2). Below that value, only stimulation is apparent (in the range pH 5.3—7.4). As the pH rises, the value for maximum stimulation decreases and shifts toward the region of lower cellobiose concentrations.

Other sugars have been tested for their effect on hydrolysis of CMC in the viscosimeter. The conditions chosen were those which proved optimal for

Figure 3. *Effect of cellobiose concentration on stimulation and inhibition at pH 5.3* (CMC — Fluidity tests).

- I *Myrothecium verrucaria*
- II *Streptomyces sp.*
- III *Trichoderma viride*
- IV *Penicillium pusillum*



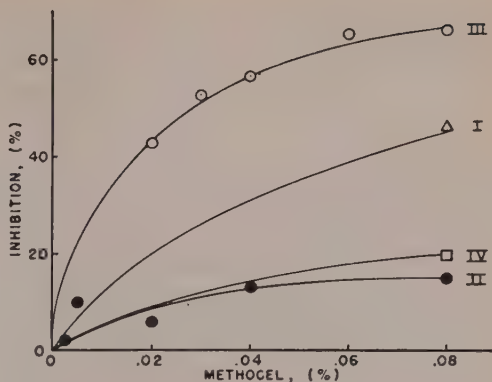
cellobiose. Sucrose, glucose and maltose were tested with each of the four filtrates under comparison. In only one case, was there any significant effect, — and here the effect was opposite to that observed with cellobiose. The *Trichoderma viride* filtrate, normally inhibited by cellobiose, was stimulated by glucose (maltose and sucrose had no effect). The effect is slight (15–20 per cent), greatest at a glucose concentration of 2 percent, and is relatively independent of pH.

Methocel (Methyl cellulose, DS 1.8) is a water soluble cellulose derivative of high degree of substitution. As a result, it is resistant to hydrolysis by cellulolytic enzymes. Preliminary tests had shown methocel to be very effective in inhibiting Cx. It was now retested with the four filtrates under consideration (Figure 4). All four filtrates were inhibited by methocel, but to greatly varying degrees. Most sensitive was that of *Trichoderma viride*, followed by *Myrothecium verrucaria*, *Penicillium pusillum* and *Streptomyces sp.* This is exactly the same order previously observed for adsorption of the enzyme Cx of these filtrates on a purified wood cellulose (unpublished). In the latter tests, the Cx was found to be adsorbed much more firmly than was β -glucosidase, from which it is inferred that enzyme-substrate attraction was involved. It has been shown, however, (Teragama *et al.* 1950) that cellulose sulfate inhibits catalase activity. The methocel in our experiments may be acting in a similar non-discriminatory manner.

Since carboxymethyl cellulose is a moderately strong acid, there is a tendency to explain any differences observed in behavior between it and cellulose on the basis of the characteristics of the acidic group. This would be particularly so in explaining the effect of pH on cellobiose stimulation (or

Figure 4. Inhibition of CMC hydrolysis by methocel (Viscosimetric Method).

- I *Myrothecium verrucaria*
 II *Streptomyces sp.*
 III *Trichoderma viride*
 IV *Penicillium pusillum*



inhibition) of the hydrolysis rate. We have, therefore, repeated some of the above experiments using non-acidic water soluble cellulose derivatives:

Cellulose acetate (Eastman Kodak Co.) DS 0.76

Hydroxyethyl cellulose (Carbide and Carbon Chem. Co.)

With the cellulose acetate, hydrolysis by the *Streptomyces sp.* filtrate was stimulated by cellobiose; hydrolysis by *Penicillium pusillum* filtrate was inhibited by cellobiose. With the hydroxyethyl cellulose, cellobiose stimulation of hydrolysis by *Streptomyces sp.* filtrate was greatest at the lower pH values and decreased as the pH became higher. At pH 7.5, maximum stimulation was found at 0.7 per cent cellobiose; inhibition occurred above 2.5 per cent cellobiose. All of these data are in agreement with those obtained using carboxymethyl cellulose as substrate. The effects reported would seem to be independent of the nature of the substituent present.

(2) Hydrolysis of unsubstituted cellulose

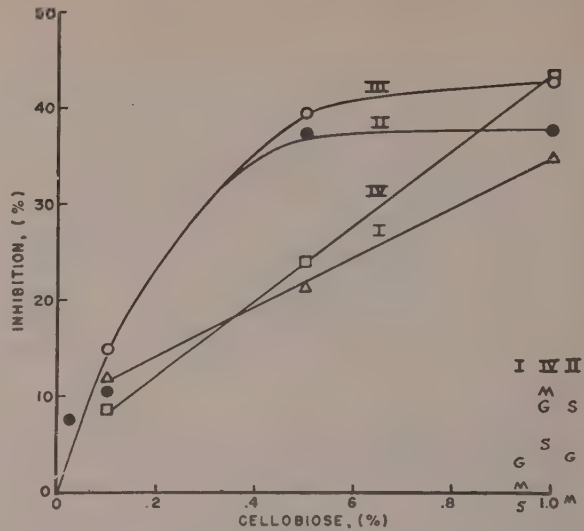
Hydrolyses of both swollen cellulose (Figure 5), and of ground cotton fiber (Table 2), are inhibited by the addition of cellobiose. All filtrates behave in the same manner. No stimulation was observed at any time.

In the swollen cellulose tests, glucose, maltose, and sucrose were compared with cellobiose at the 1 per cent level. The results, for the sugars other than cellobiose, are somewhat erratic because the weights are so near those of the controls. The maximum values for inhibitions by a 1 per cent concentration of glucose, maltose, or sucrose are of the magnitude obtained for the action of 0.1 per cent cellobiose (Figure 5). Six other filtrates (in addition to those shown in Figure 5) were tested on swollen cellulose. Three of these, *Aspergillus flavus*, *Torula sp.* and *Scopulariopsis brevicaulis*, were inhibited by cellobiose but not by glucose. Of the others, that of *Sporotrichum pruinosum*

Figure 5. Inhibition of cellulolytic activity by cellobiose (loss in weight of swollen cellulose).

- I *Myrothecium verrucaria*
- II *Streptomyces* sp.
- III *Trichoderma viride*
- IV *Penicillium pusillum*

Effects of other sugars at the one percent level.
M = Maltose
G = Glucose
S = Sucrose



produced very little loss in weight, while those of *Pestalotia palmarum* and of *Botrytis cinerea* were inhibited to a slight but equal extent by both cellobiose and glucose. Similar, but more limited data were obtained with cotton cellulose used as substratum. The inhibition, thus, appears to be specific for cellobiose.

Methocel was employed in but one test with cotton fiber as substratum. The filtrate selected (*Trichoderma viride*) was that whose Cx was inhibited most in the CMC experiments. For this set of conditions, 0.01 per cent metho-

Tab. 2. Effect of cellobiose on hydrolysis of cotton cellulose by cell-free filtrates.

Filtrate of	QM No.	Cellobiose Conc. %	pH	Loss in Wt. % ²		Inhibition, % ¹
				No CB	+CB	
<i>Myrothecium verrucaria</i>	460	4.	5.2	7.6	3.8	50
		4.	6.4	8.3	2.9	65
<i>Penicillium pusillum</i>	137 g	0.4	5.2	8.8	5.0	43
		1.0	5.2	8.8	3.0	66
		5.0	5.2	11.5	3.7	68
<i>Streptomyces</i> sp.	B814	1.	5.7	6.9	2.7	61
		4.	5.7	6.9	1.45	79
<i>Trichoderma viride</i>	6 a	1.0	5.2	7.4	2.2	70
		5.0	5.2	4.6	0.5	89

¹ Hydrolysis measured as loss in weight of cotton cellulose.
² % Inhibition = $\frac{(\text{loss in absence of CB}) - (\text{loss in presence of CB})}{\text{loss in absence of cellobiose}} \times 100$.
² Four replicates for each condition.

cel gave a 55 per cent inhibition of hydrolysis as compared to a 57 per cent inhibition by 1 per cent cellobiose. Methocel is, then, about 100 times as active as cellobiose in inhibiting this particular enzyme.

Discussion

For purposes of convenience, the stimulatory effect of cellobiose will first be considered, followed later by a discussion of the inhibitory effect. The stimulation by cellobiose of the Cx activity of *Streptomyces spp.* filtrates takes place only when the substrate is a form of cellulose solubilized by the introduction of various substituents (CMC, hydroxyethyl cellulose, cellulose acetate). When unsubstituted cellulose is used the stimulation is absent. The effect is of significance here only as it may lead to some understanding of the action of enzymes on modified substrates. The facts regarding the *Streptomyces* filtrate are:

- (1) Cellobiose stimulation of Cx at low pH
- (2) Cellobiose inhibition of Cx at high pH
- (3) Methocel inhibition of Cx

Because of the similarity of cellobiose and methocel to the natural substrate (i.e. cellulose), we assume that they compete with the CMC for the active sites on the enzyme Cx, i.e. they all have an affinity for the same loci. When CMC occupies the active position, it remains there until hydrolysis occurs, and the products are then removed. Cellobiose occupies the site for the least time, the natural tendency being to thrust off the end products. Methocel, however, is retained longer at the locus since it resists hydrolysis. Now it is assumed that the configuration of the Cx of *Streptomyces sp.* is a bit different from the Cx of most other organisms studied in that the hydrolysis products of the CMC are not as readily removed from the active position. The result is that the enzyme is unable to combine with another molecule of substrate. It is then possible that cellobiose, competing for the same areas, aids in the removal of the refractory products. This explanation does not consider the very marked effect of pH on cellobiose stimulation of *Streptomyces sp.* filtrates. For a constant cellobiose concentration the stimulation is greatest at the lower part of the pH range. It may be that in this region the binding force of the refractory-CMC-products to the enzyme is greatest, since other tests have shown that adsorption of Cx on cellulose increases with increasing hydrogen ion concentration. On the other hand, the fact that stimulation is greatest at low pH may also be explained on the basis of cellobiose protection of Cx by preventing enzyme inactivation. Similar protective actions have been reported for

- (1) yeast invertase by protein at low (but not at the optimum) pH
- (2) invertase by sucrose
- (3) α amylase by starch and its hydrolysis products.

The objection to this »protective» supposition is that the stimulation is not apparent when *unsubstituted* celluloses (cotton, swollen cellulose) are used as substrates.

We will now consider the *inhibitory* action of cellobiose. Cellobiose inhibits the Cx of most of the organisms studied. Even in those cases where stimulation is observed at low cellobiose concentrations (i.e. with CMC), the inhibitory effect becomes evident as the cellobiose concentration is increased. Since other sugars have little or no effect, the specificity of the reaction lends support to the belief that cellobiose is the end product of the enzyme (Cx) hydrolysis. This is confirmation of earlier work reporting the presence of cellobiose in hydrolysates of soluble cellulose derivatives (Levinson *et al.* 1951).

The inhibition of a reaction by its end products may lead to a better understanding of the nature of the enzyme action. For purposes of comparison, the paucity of data on the effect of maltose on β -amylase and on α -amylase is unfortunate. It appears (Schwimmer 1950) that maltose inhibits α -amylase in a non-competitive manner, but that hydrolyzable dextrans act competitively. This is consistent with the theory of random action coupled with the greater affinity of this enzyme for the longer chains. No data of this kind have come to our attention regarding the effect of maltose on β -amylase, though it has long been known (Kühn 1925) that maltose does inhibit β -amylase. It might be argued (in the absence of data) that the inhibition should be competitive since maltose results from each hydrolytic act of the β -amylase. Our data show that cellobiose specifically inhibits the Cx activity of most, but not of all the microorganisms that we have examined. Based on meager CMC results (unreported), it appears that cellobiose usually acts non-competitively, similar in effect to maltose on α -amylase. This is in agreement with the earlier data supporting random splitting of the cellulose chain:

- (1) Fåhraeus (1946): Cellophane strips lost rapidly in tensile strength before any glucose could be demonstrated.
- (2) Norkrans (1950): Rapid depolymerization of regenerated cellulose occurred without a corresponding increase in sugar formation.
- (3) Levinson and Reese (1950): A rapid loss in viscosity of CMC per unit increment in reducing value was found in the early stages of hydrolysis.

Cellobiose inhibits cellulolytic action as measured by loss in weight of native cotton, as well as that measured by loss in viscosity of CMC. This

observation must be examined for its effect on the two step mechanism postulated for the breakdown of cellulose.



We believe that it is the action of C_x on *insoluble* fragments (resulting from C_1 activity) that leads to the losses in weight which have been measured.

We have considered following farther the comparison of C_x with the amylases. β -amylase acts on starch to produce β -maltose, its action proceeding from the non-reducing end of the molecule. Why the configuration is β rather than α is as yet unknown. α -amylase splits the chains in a random fashion, the final product being α -maltose. Where the hydrolysis reaction proceeds rapidly, it is possible to detect the α or β form of maltose, and thus to determine whether the amylase is of the α or β type. To date, we have been unable to find a substrate of suitable nature to permit of C_x activity of the magnitude required. An activity resulting in the production of 0.3 per cent cellobiose within 30 minutes would probably be sufficient to permit detection of the α or the β form of cellobiose, before mutarotation resulted in the establishment of the equilibrium mixture.

Further comparison of C_x with amylases forces us to recognize that the characterization of fungal amylases as α -type may no longer hold. It now appears (Okazaki 1950) that both α and β amylases are present in the same fungus. The possibility that there are similarly two (or more) C_x types in each organism must not be overlooked. The bimodal pH-activity curves support such a view. While the evidence for such mixtures of C_x types is scanty, this may be due entirely to insufficient experimentation. Most of our present data, however, support the view that the filtrates of a fungus contain one C_x type acting in a random fashion similar to α -amylase.

Summary

Cellobiose inhibits the hydrolysis of cellulose by filtrates of most of the organisms tested. Glucose does not inhibit the hydrolysis. The action of cellobiose is believed to be that of an end product inhibiting an enzymatic hydrolysis, in much the same manner that maltose inhibits hydrolysis of starch by α -amylase.

Filtrates of seven organisms (of 36 tested) have been found where the hydrolysis of carboxymethyl cellulose is *stimulated* by low cellobiose concentrations. As the cellobiose concentration was increased, the stimulation decreased, and finally inhibition occurred. The hydrolysis of *unsubstituted* cellulose by these same filtrates is inhibited by cellobiose.

References

- Fåhræus, G.: Enzyme preparations from cellulose-decomposing bacteria. — *Experientia* 2: 413. 1946.
- Hanes, C. S. & Cattle, M.: Starch-iodine coloration as an index of differential degradation by the amylases. — *Proc. Roy. Soc. London, Series B*: 125: 387. 1938.
- Karrer, P. & Staub, M.: Polysaccharide XXVII. Zur Kenntnis der Lichenase. — *Helv. Chim. Acta* 7: 916. 1924.
- Karrer, P., Staub, M., Weinhausen, A. & Joos, B.: Zur Kenntnis der Lichenase und Reserve-cellulose (Lichenin). — *Helv. Chim. Acta* 7: 144. 1924.
- Kühn, R.: Der Wirkungsmechanismus der Amylasen; ein Beitrag zum Konfigurationsproblem der Stärke. — *Liebigs Ann.* 443: 1. 1925.
- Levinson, H. S., Mandels, G. R. & Reese, E. T.: Products of enzymatic hydrolysis of cellulose. — *Arch. Biochem. and Biophys.* 31: 351. 1951.
- Levinson, H. S., & Reese, E. T.: Enzymatic hydrolysis of soluble cellulose derivatives as measured by changes in viscosity. — *J. Gen. Physiol.* 33: 601. 1950.
- Lockwood, L. B.: Personal communication. 1952.
- Mansour, K., & Mansour-Bek, J. J.: On the cellulase and other enzymes of the larvae of *Stromatium fulvum*. Villers (Family Cerambycidae) — *Enzymologia* 4: 1. 1937.
- Norkrans, Birgitta: Studies in growth and cellulolytic enzymes of *Tricholoma*. — *Symbolae Botanicae Upsaliensis* 11 (1): 1. 1950.
- Okazaki, Hiroshi: Amylases of various species of molds. I. Classification of molds into five types after the manner of starch hydrolysis. — *Jour. Agric. Chem. Soc. Japan* 24 (2): 88. 1950. (Seen only in abstract).
- Reese, E. T., Siu, R. G. H., & Levinson, H. S.: Biological degradation of soluble cellulose derivatives. — *J. Bact.* 59: 485. 1950.
- Schwimmer, S.: Kinetics of malt α -amylase action. — *J. Biol. Chem.* 186: 181. 1950.
- Teragama, H., Yamaha, T., & Kusama, K.: Enzymic activity of catalase-high molecular electrolyte complexes. — *J. Chem. Soc. Japan (Pure Chem. Sec.)* 71: 219. 1950.
- Walseth, C. S.: Enzymatic hydrolysis of cellulose. — Thesis (PhD) presented to Inst. of Paper Chemistry, Appleton, Wisc. 1948.

Hans Fitting zum 75. Geburtstag gewidmet

Studies on Growth and Metabolism of Roots. VIII. Calcium as a Growth Factor

By

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It has become necessary in the course of investigations of the auxin and antiauxin actions on root growth to recognize the importance of the pH of the nutrient medium for root elongation. This is complicated by the fact that conflicting statements are found in the literature concerning even the general shape of the growth-pH curves, not to mention the interpretations.

Lundegårdh (13) and Audus (1) both found growth-pH curves with a maximum at pH 5 to 6, but some of our experiments (7) gave a steadily increasing root length with a pH increasing from 4 to 7. Still other relationships can be found in the literature. It is customary in current research to interpret the growth-pH curves as due to the dissociation of native auxin. References to earlier papers are found in the two quoted works, and it will therefore suffice to compare the different opinions expressed by these authors. An increase in growth from the acid side to pH 5 or 6 has been explained by Audus and other investigators as due to a decreasing excess of growth inhibiting auxin acid molecules, but by Lundegårdh as due to a positive action of auxin anions. The decreasing growth from pH 6 to neutral reaction again is explained by Audus by the assumption that the concentration of undissociated acid is suboptimal and increases with increasing pH. It has further been shown (7) that the activity of antiauxins, presumed to antagonize native auxin, follows the concentration of antiauxin anions.

Owing to this unsatisfactory situation it was deemed necessary to reinvestigate the problem of the growth-pH relationship with the aim of determining the cause of at least the experimental discrepancies. Experiments have thus been carried out on wheat with routine methods for growing plants in continuously flowing solutions (5, 20) with varied pH values. The root growth was followed for three days at a temperature of 23—25° C. The pH of the solutions was varied with potassium phosphates, and the calcium content with calcium chloride. The basic nutrient solu-

tion contained KNO_3 1/2000, MgSO_4 1/4000, and PO_4 1/1000 M . — All concentrations in the text and figures are expressed in molarity. The plant material as in previous experiments was »Eroica» winter wheat, and in one instance barley, »Gullkorn».

Interaction of Ca and H

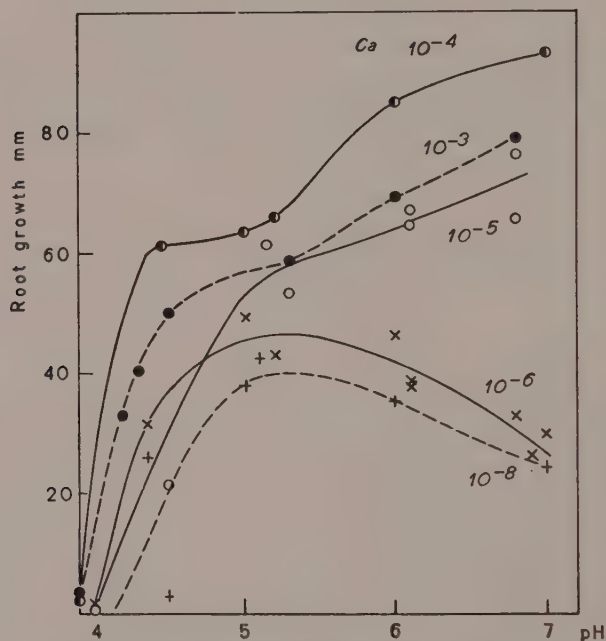


Figure 1. The relation between pH and root elongation at different calcium levels.

It was easily established that the shape of the root-elongation-pH curve varies with the concentration of calcium in the medium in the way illustrated in figure 1. This graph is a compilation of a number of experiments carried out during a period of about five months. The reproducibility of the experiments was satisfactory, although some irregularities occurred particularly at pH values below 4.5. The results can be summarized as follows. The optimum curve for root elongation with a maximum between pH 5 and 6 is obtained at calcium concentrations up to $10^{-6} M$. An increase to 10^{-5} causes the drop toward neutral reaction to disappear, giving a curve with a steep rise from pH 4 to 7. A further increase to 10^{-4} counteracts the growth inhibition also at pH 4.5 and gives from this value to pH 7 the convex curve found

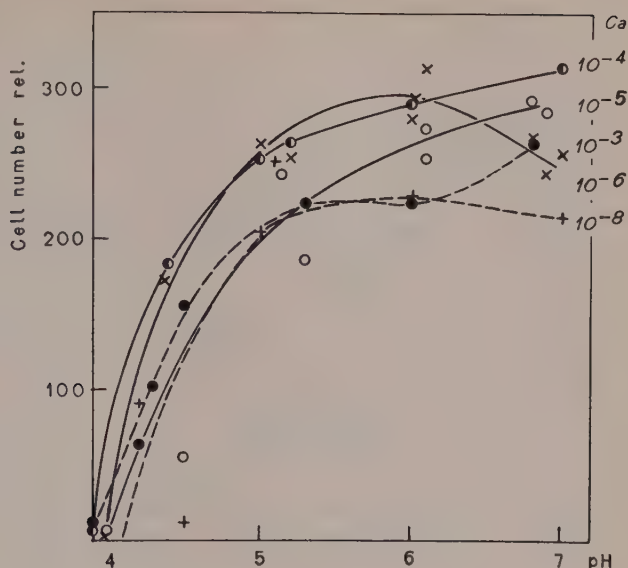


Figure 2. *The relation between pH and cell multiplication at different calcium levels. The same experiments as in figure 1.*

in our previous experiments. Calcium 10^{-3} M is already supraoptimal. In this way a series of growth-pH curves can be obtained obviously imitating the various forms already described in the literature. Solutions containing Ca only as a contamination permitted a growth up to three days fairly similar to that with Ca 10^{-8} , but after four days the roots usually died. This shows that the minimum limit of Ca lies near 10^{-8} M.

These growth curves were analyzed with respect to the two modes of growth, those by cell multiplication and cell elongation. The results are shown in Figures 2 and 3, and they give an entirely different picture of the interaction between calcium and hydrogen ions on the root growth.

The curves illustrating the relation between pH and cell multiplication are of a simple shape (Figure 2). Most noticeable is the slight effect of calcium. Already at a concentration of 10^{-6} M there is a maximal rate of cell multiplication, and this content does not correspond to more than 0.04 p.p.m.

The relation between pH, Ca and cell elongation at first seems to be more complicated. Starting from the general growth curve with a low calcium concentration it is appropriate, however, to divide the diagram in two parts, below and above a pH of approximately 5, and deal with them separately. This is also justified concerning the cell multiplication; it follows from Figure 2 that above pH 5 there is a very slight increase in the cell multiplication, but below that value a rapidly decreasing rate with decreasing pH.

This break at pH 5 is still more pronounced with regard to the cell elongation. Up to calcium 10^{-6} and with a pH below 5 the cell length figures are

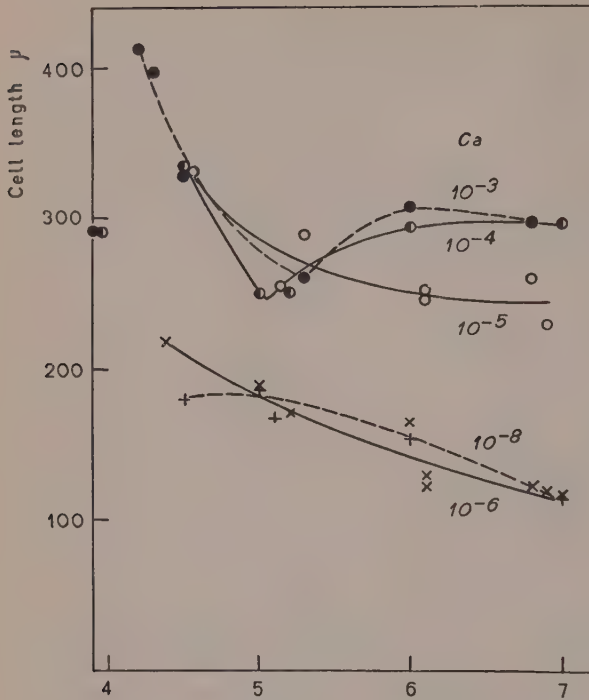


Figure 3. The relation between pH and cell elongation at different calcium levels. The same experiments as in figure 1 and 2.

low but fairly constant; from pH 5 onwards the elongation steadily decreases. The step from calcium 10^{-6} to 10^{-5} M, however, increases the cell elongation to top figures in the most acid solutions, and the cell lengths decrease rapidly with a pH increasing to 5. In the range of pH 5 to 7, on the contrary, the elongation becomes more or less independent of the pH. It should also be noted that calcium 10^{-3} is not supraoptimal for the cell elongation, but tallies closely with 10^{-4} M.

In summing up these results the bulk growth curve can be dissected in the following way. The decreasing root elongation on the acid side of pH 5 depends only upon a decreasing cell multiplication, which at a pH near 4 leads to the death of the roots. This is obviously a kind of toxicity of the solutions, increasing with increasing H-ion concentrations. It is counteracted at pH 4.5 and higher values by Ca in a concentration of 10^{-5} to 10^{-4} M. The toxicity of acid solutions is a well-known phenomenon, and it is generally ascribed to the action of the H-ions themselves, causing a destruction of the cytoplasm. Nothing contradicts the assumption that this is what is encountered in the present experiments, and the great cell lengths in the most acid solutions are inconsistent with the assumption of a toxic action of an auxin excess, which has been brought up in order to explain the decreasing growth

in acid solutions. It is true that Lundegårdh (13) also on wheat roots found the toxic action of H-ions near pH 3, but it was in short-term tests, and like other toxic actions that of H-ions ought to involve a time factor. The counter-action by Ca-ions is also well known from the extensive literature on ion antagonism and the detoxicating action of especially Ca-ions; no reasons are found in the histological behaviour of the roots, which support the idea that auxin is in any way responsible for the action of the H-ion concentration on the roots within this range of pH.

The decreasing growth above pH 5 and with a low Ca concentration depends entirely upon a decreasing cell elongation. This is what might be expected, if it were caused by either a decreasing deficiency of auxin in suboptimal concentration, or an increasing concentration of an inhibitor present in excess. In both cases mainly or only the cell elongation, but not the cell multiplication, is affected (Burström 4, 8). It is surprising to find, however, that even this growth depression is counteracted by an increase in the Ca concentration, so that with optimal Ca the cell elongation is fairly independent of the pH. The slowly increasing cell multiplication is then responsible for the rise in the general growth curve with increasing pH.

Interaction of Ca and Auxins

It is difficult to make the supposition that calcium directly antagonizes auxin, if this is the growth factor responsible for the shape of the growth curve. One way to tackle this problem is to find out, how Ca behaves towards a real auxin antagonist, and whether this can reveal the cause of the decreasing growth in neutral solutions. The antagonist chosen was parachlorophenoxy-*iso*-butyric acid (PCIB), and its interaction with Ca has been studied.

This was accomplished in a series of experiments, one of which is recorded in Figures 4 and 5. The plan is evident from Figure 4. High and low Ca concentrations were combined with the absence and presence of PCIB 10^{-5} M, which gives the maximal growth effect. The preparation of the latter contained according to analyses ca. 10^{-8} M Ca on 10^{-5} M acid, which does not invalidate the results, however, since it appears from Figure 3 that variations in the Ca concentration between 10^{-8} and 10^{-6} do not visibly affect the cell elongation. The data for the cell multiplication have not been recorded, because they only confirm previous experiences of the mode of action of both growth factors.

Figure 4 shows that PCIB exerts its maximal effect at pH 5 and Ca at pH 7, when added separately; when the antiauxin is added to the high Ca

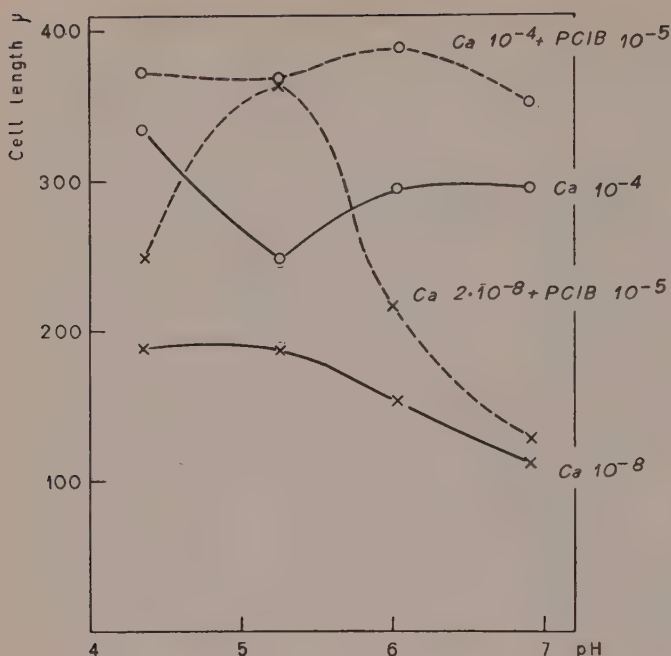


Figure 4. The relation between pH and cell elongation at two levels of each calcium and the antiauxin PCIB.

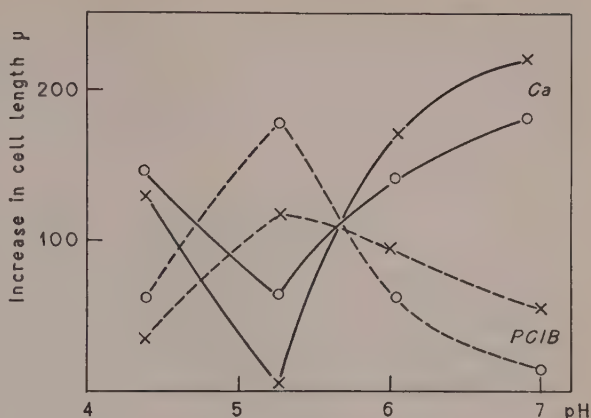
concentration the cell elongation is uniformly raised to a high level, and the sensitivity of the roots to the H-ion concentration disappears.

It is especially interesting to trace an interaction between calcium and the antiauxin, and for that purpose Figure 5 has been constructed showing the effects of the additions of the compounds. Two facts are conspicuous. Firstly, that each compound has principally one effect, irrespective of whether the concentration of the other one is high or low, which implies that the actions are approximately synergistic. Secondly, that the effects of calcium and PCIB

Table 1. The interaction between calcium and PCIB on the cell elongation in barley roots. — Increase in cell length caused by the addition of PCIB 10^{-5} M or an increase in Ca concentration from 10^{-6} to 10^{-3} M. The experiment corresponds to that with wheat recorded in figure 5. Mean error ± 4 μ .

Addition		Increase in cell length μ at pH		
		5.3	6.1	6.9
Ca	no PCIB	64	101	118
	with PCIB	62	130	169
PCIB	low Ca	80	32	9
	high Ca	78	61	60

Figure 5. *The separate actions of calcium and PCIB in the experiments of figure 4.* The ordinate shows the increase in cell length caused by an increase in calcium or PCIB content. For calcium open circles denote the effect without PCIB, crosses with PCIB present; for PCIB the circles denote low calcium content, crosses high content.



are the reverse with respect to the pH, high activity of calcium corresponds to a low activity of PCIB and conversely.

This experiment was repeated with barley instead of wheat (Table 1). The result is principally the same, although barley has a somewhat different growth-pH curve. For instance, pH below 5 is under all circumstances fatal to barley roots. Nevertheless, the general actions of Ca and PCIB are the same as with wheat, increasing effect of Ca and decreasing effect of PCIB with increasing pH.

It follows logically from these results that there must also exist an interaction between calcium and externally added auxin, and since Ca and anti-auxin act synergistically, calcium and auxin should be antagonists. This is also shown to hold true by the experiments recorded in Table 2.

The activity of Ca alone is that already described; it is antagonized by NAA in a slight excess and the entire effect of calcium on the elongation

Table 2. *The interaction between calcium and naphthylacetic acid (NAA) on root growth.* pH 5.3–5.9.

In the solution M		Increase in root length mm	Cell length μ	Relative cell number	Root hairs
Ca	NAA				
ca. 10^{-8}	0	45 ± 1.0	238 ± 4	190	Poor, < 0.05 mm.
10^{-4}	0	70 ± 1.2	345 ± 5	204	Excellent, 0.5–0.8 mm.
10^{-3}	0	65 ± 1.3	342 ± 4	191	» »
10^{-2}	0	68 ± 1.2	339 ± 4	201	Good, 0.3–0.7 mm.
ca. 10^{-8}	10^{-7}	36 ± 1.0	193 ± 3	189	Good, about 0.25 mm.
10^{-4}	10^{-7}	37 ± 1.0	178 ± 3	210	Excellent, 0.7–1.0 mm.
10^{-3}	10^{-7}	33 ± 0.9	169 ± 3	194	» up to 1.3 mm.
10^{-2}	10^{-7}	28 ± 1.0	163 ± 3	174	Very irregular, partly > 1 mm, partly lacking.

disappears. Superficially the roots, living and healthy as illustrated by the data of the cell multiplication, have become insensitive to calcium, but it is interesting to note that the favourable effect on the root hair formation is still maintained. This must obviously be treated as a separate problem.

Calcium as a Growth Factor

It has long been established that calcium has a specific importance for roots (see, e.g. the extensive investigations by Mevius 15, 16; Aulich 2), and it is rather surprising that this circumstance has been neglected in the recent growth literature. It seems, however, as if the successful introduction of the auxin conception has led to an exaggeration of the hormonal side of the growth and a somewhat prejudiced treatment of the problems. It was already shown by Hansteen Cranner (11) that calcium must be continuously present in the nutrient medium; the same has recently been demonstrated by Haynes and Robbins (12). The interaction between calcium and growth hormones has, as far as the writer is aware, only been studied by Wuhrmann (21) on coleoptiles, and his results can hardly be applied to the specific conditions prevailing in roots.

The actual Ca requirement of wheat is exceedingly low. Maximal cell multiplication, at least at pH 5 to 6, is brought about by Ca 10^{-6} M, which equals 0.04 p.p.m., or far below the optimum content of most micro nutrient elements. It should be borne in mind, however, that the seedlings feed partly on the seeds, so that no definite conclusions can be drawn as to the exact requirement of calcium as a nutrient. The most obvious effect of calcium is, however, that of a cell elongation factor, produced by Ca about 10^{-5} , or 0.4 p.p.m. The requirement is probably higher in a third respect, that is as a detoxicant in acid solutions. Mevius (16) has stated that the extreme calciphobe *Pinus Pinaster* has a calcium optimum around 10^{-5} M. Two other types of plants have been studied by Olsen (17), *Dianthus*, forming oxalate, with a calcium optimum around 10^{-4} M, and *Sinapis* lacking oxalate with a rapid increase in growth up to Ca $5 \cdot 10^{-4}$ M and an ensuing low one to 10^{-2} M. *Hordeum* and *Tussilago*, both preferring a high pH of the medium have high calcium optima (Olsen 18), just as pea (Sorokin and Sommer 19). *Triticum* apparently represents another type again, a lime-enduring but not calciphilous, non-oxalate accumulating, plant, perhaps best characterized as a silicon plant, with a calcium requirement nearly as low as that of a genuine calciphobous species.

The action of Ca has obviously a minimum around pH 5. The rise towards a more acid reaction is probably part of the detoxicating action, but the

increasing activity towards pH 7 requires an explanation. The general phenomenon agrees with that described by Mevius (15) for *Pinus Pinaster*, which exhibits a slow growth at an acid reaction, independent of Ca, but at a higher pH shows a response to Ca additions.

Calcium has at these pH-values undoubtedly the character of a positive cell elongation factor, and so has the antiauxin, PCIB; it can furthermore hardly be a mere coincidence that the activity curves of the two compounds have the reverse shape. PCIB has been supposed to act through antagonizing native auxin, an assumption which is borne out by its histological effects (7). High activity of a given amount of added antiauxin should then imply that the auxin content or the activity of the present native auxin under such circumstances were low, but, nevertheless well above the optimum. This should be the case at pH 5, and the native auxin content or activity should decrease with increasing pH. This tallies nicely with the fact that the elongation curve at a low calcium content decreases with increasing pH, indicating an increasing excess of growth inhibiting auxin. If this is caused by the dissociation of auxin this must be active in its ionic form, but such has been assumed to be the case the with antiauxins (7). If, on the other hand, the decreasing elongation towards pH 7 depends upon a deficit of auxin caused by a decreasing content of undissociated auxin, it is difficult to explain that PCIB, which antagonizes at least externally added auxin, could increase the elongation with increasing content of active auxin acid molecules.

Thus it might be assumed that native auxin, like antiauxins, are active in the anionic form, and that the decreasing elongation at neutral reaction depends upon an increasing ion content. If this assumption is wrong there seems to be no possibility at all of explaining any part of the growth-pH curve as due to the dissociation of auxin, and this may even be a sounder approach to the problem.

However, it does not explain the calcium action. It is maximal when the auxin content is supposed to be high, and almost nil when the presumed low content at pH 5 is decreased by an antiauxin addition. This indicates that calcium and auxins — including antiauxins — act on the same elongation mechanism. The primary action of auxin has not been established. It is obvious, however, that the elongation constitutes principally an increase in volume by the absorption of water, the »auxin-induced water uptake». According to the general picture of the osmotic conditions of cells this can be caused by an increased internal osmotic value, a water absorption due to forces such as electro-osmosis, or to a change in the wall pressure.

The first possibility can apparently be ruled out (10) even for roots (3). That the second mode of water absorption could attain such a height as to explain the normal elongation process has not at least been supported by

experimental evidence hitherto. Both these possibilities would imply a mere passive stretching of the cell wall, which does not seem to hold true of the root elongation (4). Directly or indirectly the auxin and antiauxin regulated elongation apparently involves changes within the cell wall (6). It is then pertinent to recall that calcium forms an integral constituent of the primary cell wall, or generally the pectic substances of the wall. The interdependence of calcium and auxin may depend upon some mutual interaction in the formation or stabilization of the wall.

According to one opinion the effect of calcium consists in a hardening of the cell wall, leading to shorter cells, which are forced to produce root hairs on the surface of the root (Cormack 9). There is no reason in this connexion to dwell upon the special problem of root hair formation, but Cormack's views are of more general interest. It was concluded by the author (4) that if the auxin action generally consists in a dissolving or softening of the cell wall, this could explain also the growth inhibition, provided that the dissolving also involved a prevention of the building up of new parts of the cell wall. It is in agreement with this idea that calcium counteracts auxins and hardens the cell walls. What does not tally is that Cormack has made use of this principle in order to explain the shortening of cells in connexion with the calcium induced root hair formation, whereas the present writer concludes that calcium increases the cell elongation.

This discrepancy can be explained tentatively on the basis of the theory of the reverse action of auxin on the first and the second parts of the cell elongation (4, 6). During the first phase, when root hairs are initiated, auxin dissolves the cell wall, and elongation takes place without a new formation of wall material. Calcium then acts as assumed by Cormack, antagonizing auxin. During the second phase the elongation depends upon a deposition of new wall material, which is prevented by auxin, and the action of calcium is reversed accordingly. — It ought to be noticed that with regard to the effects of the H-ions Cormack's and the present results tally very well.

Summary

The interaction of calcium, H-ions and an antiauxin (PCIB) on root elongation has been studied on wheat and barley roots.

The growth-pH curve changes its shape regularly under the influence of varying calcium concentrations. At an external calcium content up to 10^{-6} M root elongation in wheat has an optimum at pH 5 to 6, at higher concentrations it continuously increases up to pH 7. The decrease towards acid reaction is caused by a toxic action antagonized by calcium; it does not resemble histo-

logically a growth inhibition caused by auxin. At pH 6 calcium acts as a genuine cell elongation factor, its action adds itself to that of the antiauxin. With low calcium the cell elongation steadily decreases with increasing pH. PCIB exerts its maximal effect at pH about 5, and this runs inversely to that of calcium.

It is concluded that the only part of the normal growth-pH curve, which can be interpreted in terms of auxin content or auxin activity is the decreasing growth with increasing pH at a low calcium content; it might depend upon an excess of growth-inhibiting auxin, which increases with the pH, but it is even more likely that no part of the root growth-pH curve depends directly upon the auxin activity.

The function of calcium as an elongation factor has been discussed, and a tentative explanation is given of the interference with auxin, generally involving that auxin dissolves and calcium hardens the cell wall.

References

1. Audus, L. J.: Studies on the pH-relationships of root growth and its inhibition by 2,4-dichlorophenoxyacetic acid and coumarin. — *New Phytol.* 48: 97. 1948.
2. Aulich, K.: Beiträge zur Kenntnis der Calcium-Wirkungen auf das Keimlingswachstum von Weizen. — *Ber. Schweiz. Bot. Ges.* 45: 335. 1936.
3. Burström, H.: Die osmotischen Verhältnisse während des Streckungswachstum der Wurzel. — *Lantbr. Högsk. Annal.* 10: 1. 1942.
4. — The influence of heteroauxin on cell growth and root development. — *Ibid.* 10: 209. 1942.
5. — Studies on growth and metabolism of roots. IV. Positive and negative auxin effects on cell elongation. — *Physiol. Plant.* 3: 277. 1950.
6. — Mechanisms of cell elongation. — *Skoog: Plant growth substances*, p. 43. Madison 1951.
7. — Studies on growth and metabolism of roots. VI. The relative growth action of different isobutyric acid derivatives. — *Physiol. Plant.* 4: 470. 1951.
8. — Studies on growth and metabolism of roots. VII. The growth action of α (phenoxy)-propionic acids. — *Ibid.* 4: 641. 1951.
9. Cormack, R. G. H.: The development of root hairs in Angiosperms. — *Bot. Rev.* 15: 583. 1949.
10. Hackett, D. P.: The osmotic change during auxin-induced water uptake by potato tissue. — *Plant Physiol.* 27: 279. 1952.
11. Hansteen-Cranner, B.: Ueber das Verhalten der Kulturpflanzen zu den Bodensalzen. I. — *Jahrb. wiss. Bot.* 47: 289. 1910. — II. — *Ibid.* 53: 536. 1914.
12. Haynes, J. L. & Robbins, W. R.: Calcium and boron as essential factors in root environment. — *J. Amer. Soc. Agr.* 40: 795. 1948.
13. Lundegårdh, H.: Investigations as to the absorption and accumulation of inorganic ions. — *Lantbr. Högsk. Annal.* 8: 233. 1940.
14. — The growth of roots as influenced by pH and salt content of the medium. — *Ibid.* 10: 31. 1942.

15. Mevius, W.: Kalziumion und Wurzelwachstum. — *Jahrb. wiss. Bot.* 66: 183. 1927.
16. — Weitere Beiträge zum Problem des Wurzelwachstums. — *Ibid.* 69: 119. 1928.
17. Olsen, C.: Absorption of calcium and formation of oxalic acid in higher green plants. — *C. R. Lab. Carlsb. Ser. chim.* 23: 101. 1939.
18. — Water culture experiments with higher green plants in nutrient solutions having different concentrations of calcium. — *Ibid.* 24: 69. 1942.
19. Sorokin, H. & Sommer, A. L.: Effects of calcium deficiency upon the roots of *Pisum sativum*. — *Amer. J. Bot.* 27: 308. 1940.
20. Wilske, C. & Burström, H.: The growth-inhibiting action of thiophenoxy acetic acids. — *Physiol. Plant.* 3: 58. 1950.
21. Wuhrmann, K.: Der Einfluss von Neutralsalzen auf das Streckungswachstum der *Avena-Koleoptile*. — *Protopl.* 29: 362. 1937.

Inorganic Carbon Sources of Green Algae. VI. Further Experiments Concerning Photoactivation of Bicarbonate Assimilation

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Introduction

An initial inhibition of photosynthesis of algae in low concentrations of carbon dioxide, as shown by cells grown at high CO_2 concentrations, has been shown independently by Whittingham 1949 (Rabinowitch 1951, p. 908) and Österlind (1951). Although Whittingham used a strain of *Chlorella pyrenoidosa* and Österlind one of *Scenedesmus quadricauda*, it seems possible, although not certain (see below), that the same phenomenon has occurred in both cases. The explanations proposed by the authors are, however, quite different. Whittingham supposes a narcotic inhibition of the chlorophyll activity when the cells are transferred from a medium with high concentration of CO_2 to one with a low concentration and exposed to light. Österlind, on the other hand, explains the phenomenon by assuming that the HCO_3^- assimilation is inhibited when the cells are grown in high CO_2 concentrations at a high pH. When the cells are then transferred to media with low CO_2 and high HCO_3^- concentrations, the CO_2 concentration is too low for a rapid photosynthesis and the HCO_3^- cannot be used immediately. After some time, the ability to use HCO_3^- returns, and the rate of photosynthesis increases. In this paper some further experiments concerning this topic are described and discussed.

The site of the inhibited factor

Whittingham's theory is supported by his observation that if the cells after a brief exposure to light in a low CO_2 concentration are brought back

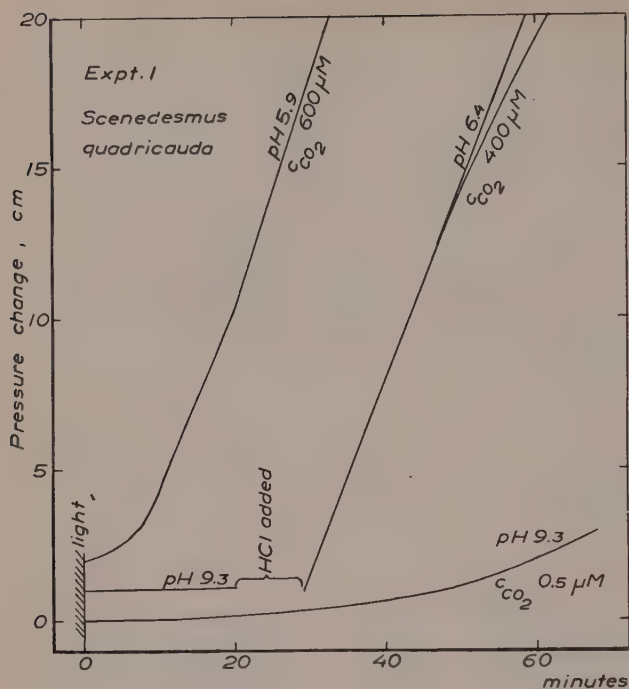


Fig. 1. Photosynthesis in inhibited cells of *Scenedesmus quadricauda*, transferred from pH 9.3 to pH 6.4.

into a medium of high CO_2 concentration, they show a reduced rate of photosynthesis in this medium as well (Rabinowitch 1951, p. 909). Some experiments to check this statement have been made, both with *Scenedesmus quadricauda* (described below) and with *Scenedesmus armatus*, but it has not been possible to obtain verification.

Expt. 1 (Figure 1). Cells of *Scenedesmus quadricauda* were used, having been grown for 5 days in a nutrient solution, aerated with 2 per cent CO_2 at pH 8.6. The cells were suspended in phosphate buffers with 1 mM total concentration of carbon sources. In addition, impurities in the phosphate buffer and, to a less degree, internal carbon sources in the cells account for 100–150 μM . In experiments of this kind, it is impossible to remove these carbon sources without destroying the inhibition.

In 2 vessels, the pH was 5.9, and in 5 vessels, 9.3. After about 25 minutes illumination, HCl was added to 3 of the latter vessels in order to increase the CO_2 concentration by decreasing pH to 6.4. At pH 5.9, photosynthesis started at once, at pH 9.3 only slowly after about 40 minutes illumination. When adding HCl, photosynthesis began immediately.

This experiment indicates that only HCO_3^- assimilation is inhibited, but not that part of photosynthesis common to both CO_2 and HCO_3^- . It may

be added that an initial inhibition of photosynthesis in high CO_2 concentrations has never been observed by the present author. It is therefore possible that the inhibition found by Whittingham is of another kind than that found by Österlind.

Kinetic experiments

Some experiments have been made in order to study the recovery of bicarbonate assimilation from a kinetic point of view. One of these experiments is reported here.

Expt. 2 (Figures 2, 3, and 4). Cells of *Scenedesmus quadricauda* were grown and treated in the same way as in Expt. 1. The pH of the phosphate buffers in the seven vessels ranged from 7.4 to 10.1. The manometer readings were begun at the start of illumination, the results being shown by the curves in Figure 2. The concentrations of CO_2 and HCO_3^- are also noted. The rate of photosynthesis has been determined every 10 minutes by estimating the inclination of the tangent at various points of the curves. These values have been used in Figure 3, showing the rate of photosynthesis at various times after the start of illumination. At the two highest pH values, photosynthesis could not be detected during the first 40 minutes, after which the rate increases, this increase being more rapid at pH 9.8 than at pH 10.1. At the lower pH values, photosynthesis starts at once but with a reduced rate, except at the lowest pH where the rate is almost constant during the entire experiment.

It is possible that a short lag period is present also in the vessels with a pH lower than 9.8, but it is not so pronounced as in the most alkaline solutions. The vessels used are not suitable for an analysis of the first part of the curves due to the low rate of diffusion from the liquid to the gas phase. After this more or less pronounced lag phase, the rate increases equally rapidly in all the vessels (with the exception of pH 10.1).

According to the theory previously proposed by the present author, these results may be explained in the following way. The initial rate of photosynthesis depends on the concentration of CO_2 . In Figure 4, the concentration of CO_2 has been compared with the rate of photosynthesis after 20 minutes. Although it would have been desirable to have a vessel with a higher CO_2 concentration, it seems certain that the rate of photosynthesis in the most acid vessel is very close to the maximum rate, since the rate does not increase when HCO_3^- becomes available to photosynthesis. At these pH values, the maximum rate is thus reached at about $60 \mu\text{M CO}_2$. In acid solu-

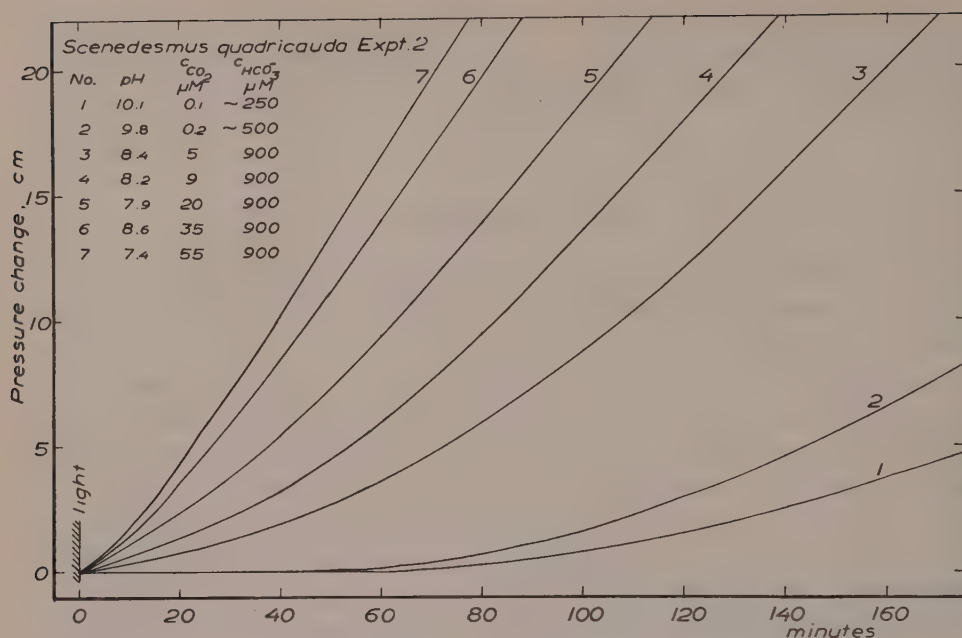


Fig. 2. Photosynthesis in inhibited cells of *Scenedesmus quadricauda* at various CO_2 concentrations.

tions (pH about 4), the maximum rate is reached at about $150 \mu M CO_2$ (unpublished results).

As was mentioned above, the rate of photosynthesis increases at the same rate in all vessels, with the exception of pH 10.1. The increase in rate is supposed to be due to the reestablishment of the ability to assimilate HCO_3^- . It may be supposed that the rate of assimilation of HCO_3^- is directly proportional to some substance, probably an enzyme, which is formed at a rate directly proportional to the time, i.e., it may be formed by a photochemical reaction. It has previously been shown that light is necessary for its formation.

According to the view of Whittingham, only CO_2 is used and the initial inhibition is due to some narcotic which has settled on the chlorophyll. As discussed previously (Österlind 1952), this presupposes that the algae assimilate CO_2 much more effectively at high pH values than at low ones. According to the theory proposed in this paper, the concentration of CO_2 necessary for maximum assimilation is only about twice as great at low pH values as at high ones. If the removal of the inhibition were related to photosynthesis (according to Rabinowitch an »autocatalytic removal»), the increase of the rate would be proportional to the photosynthetic rate, i.e., the inclina-

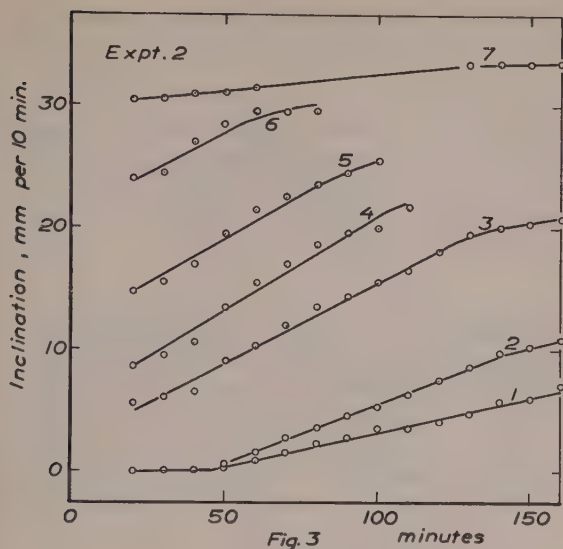
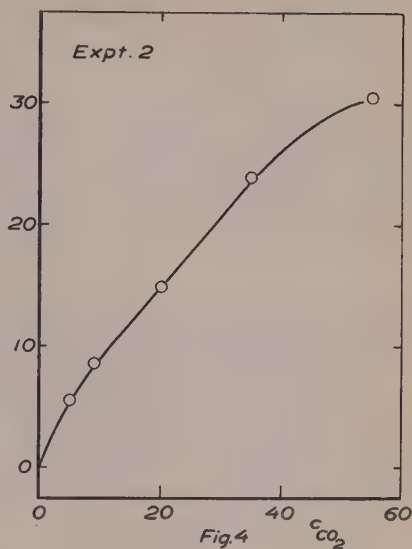


Fig. 3. The derivatives of the curves in Fig. 2.

Fig. 4. Rate of photosynthesis after 20 minutes at various CO_2 concentrations.

tion of curve 5 in Figure 3 at the start would be three times that of curve 3. Under such conditions, no straight lines would be obtained but only curves, these being convex downwards. Therefore it seems probable that the removal of the inhibition is more closely connected with light absorption than with photosynthesis.

Summary

The initial inhibition of photosynthesis of algae in low CO_2 concentrations, as shown by cells grown at high CO_2 concentration has been discussed. Two theories have been advanced; the first, proposed by Whittingham, assumes that only CO_2 is assimilated, inhibition being due to a poison settling on the chlorophyll, the second, proposed by the present author, assumes that HCO_3^- is also assimilated, inhibition being due to an inhibited absorption of HCO_3^- . At present, it is impossible to decide which of these two theories is correct, but arguments favouring the latter one have been presented by the author.

It has been shown that the CO_2 concentration necessary to produce maximum photosynthesis is slightly less in inhibited cells at high pH values than in uninhibited cells at low pH values. According to Whittingham's theory, the difference must be many times greater. It has also been shown that

during the time of inhibition, the rate of photosynthesis shows a constant increase which is independent of pH, concentration of CO_2 , and rate of photosynthesis.

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References

- Österlind, S.: Inorganic carbon sources of green algae. IV. Photoactivation of some factor necessary for bicarbonate assimilation. — *Physiol. Plant.*, 4: 514. 1951.
— Inorganic carbon sources of green algae. V. Inhibition of photosynthesis by cyanide. — *Physiol. Plant.*, 5: 372. 1952.
Rabinowitch, E. I.: Photosynthesis and related processes. Vol. II. Part 1. — New York. 1951.

On the Effect of Light and Fertilizing on the Energy Economy of Winter Wheat

By

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(Received July 8, 1952)

Introduction

Emerson has shown that the assimilation intensity of *Chlorella* depends upon its chlorophyll content, and Stålfelt that the shade needles of the spruce assimilate at $\frac{1}{2}$ day light more intensively than the light needles, of which the chlorophyll content is lower (cf. 4, p. 259). Kolisko (2), Liesegang and Remy (3), Bukatsch (1), etc. have investigated the effect of fertilizing on the pigment content and on the energy economy of plants. It is obvious from these investigations that fertilizing has a modifying effect on the assimilation mechanism of the plant, e.g. by influencing the amounts of the light absorbing pigments. Whether abundant N—P—K fertilizing can increase the ability of Varma winter wheat to utilize light energy of slight light intensity is investigated in the following. Special attention is paid also to the reasons for the variations in the dry matter percentage and top-root ratio of the plant with varying intensity and prolonged diurnal period of light (5, 6).

I have received valuable assistance in this research work from Miss Anna-Liisa Ruokola, M. Agr., and Mr. Simo Antila, M. Agr. to both of whom I wish to extend my best thanks. I am also indebted to Mr. K. Salo, M. Sc., Institute of Chemistry of the University of Helsinki, who performed the carbon, hydrogen, and ashes analyses for the following investigations.

Methods

On the bottom of Mitscherlich pots there was a 6-cm thick layer of fine sand. Above that was placed a mixture of the same sand with 100 g of field soil, and, in the fertilized pots, with an addition of 6 g of superphosphate, 3 g of 50 per cent potash salt and 12 g of CaO. On this was sown 30 seeds of wheat, and these were covered with a 3.5-cm layer of grit. 3 g of $\text{Ca}(\text{NO}_3)_2$ was sprinkled on the surface of the growth substratum soon after the sowing, and 3 g half way through the experimental period. The pots were watered often enough to keep the growth substratum moist throughout. To eliminate the differential effects of evaporation of the various pots, watering was always carried out to full water capacity. The water that ran from the pots was used for watering again. Thus, the fertilizing was very abundant, equalling per hectare: 1900 kg of superphosphate, 950 kg of 50 per cent potash salt, and 1900 kg of $\text{Ca}(\text{NO}_3)_2$.

The experiments were carried out in laboratory rooms with windows, 1.5 m high, 3 m wide, facing southeast, with the experimental pots placed at different distances from the window, the nearest at 46 cm, the others 27 cm from the preceding pots, and accordingly the last, i.e. the sixth, at 181 cm from the window. The mean light intensities for each of the pots, counted from the window, were 57, 48, 41, 36, 33 and 25 per cent of the light intensity outside the window.

To investigate the effect of the length of the diurnal period of light the pots were placed in two similar rooms. One was continuously lit with fluorescent lamps so that the night light intensity for the plants was approx. 700 lux. No artificial light was used in the other room.

In 1952 two series of experiments were carried out, the first in February—March (14.2—19.3.), the second in April (3.4—28.4).

During the February—March experimental period the length of the day and at the same time the shorter diurnal period of light was 9.1—12.0 hours. The mean temperature, in the »short photoperiod room», was 22.4°C and in »the long photoperiod room» 21.9°C. The length of day during the experimental period in April was 13.3—15.6 hours, which in the »short photoperiod room», was shortened to 10 hours. The mean temperature in »the room of the short photoperiod» was 21.1°C, and in »the long photoperiod room» 21.0°C. The content of carbon and hydrogen in the yield was analysed by Pregl's micromethod. The »remainder» of the analysis is entered in the following as ash.

Results

The period intervening between the sowing and the emergence of the wheat was the same in both of the rooms and in both series of experiments, 5 days. The fertilized plants were distinctly more green and of less erect growth than the unfertilized.

Fertilizing increased the *green shoot yield* more when the light period was long. On the green yield of the unfertilized wheat the effect of the length of the light period could not be established with certainty (Table 1). Correspondingly, the effect of the intensity of light on the green yield of the fertilized wheat was very distinctly positive, whereas its effect on the green yield of the unfertilized wheat was not discernible (Table 2).

The dry matter content of the shoots has decreased with reduced intensity of light, though more sharply in the unfertilized wheat than in the fertilized (Table 4). The positive effect of the diurnal period of light on the dry matter content, too, has manifested itself more distinctly in the unfertilized than in the fertilized wheat (Table 3). On an average the dry matter content of the unfertilized wheat has been much higher than that of the fertilized (Table 3), but with the intensity of light at its lowest (25 per cent) this difference has been hardly discernible (Table 4).

The dry matter yield of the shoots, of the fertilized wheat, on an average, has been much bigger than that of the unfertilized (Table 5), and this diff-

Table 1. *The effect of the length of diurnal light period and of fertilizing on the green shoot yield (g per 30 plants).*

Light period	Exp. 14.2.—19.3.		Exp. 3.4.—28.4.	
	Unfertilized	Fertilized	Unfertilized	Fertilized
Short	11.7	28.0	10.2	17.0
Long	9.8	35.4	12.6	23.4

Table 2. *The effect of intensity of light and of fertilizing on the amount of the green shoot yield.*

Intensity of light (°/o)	Exp. 14.2.—19.3.		Exp. 3.4.—28.4.	
	Unfertilized	Fertilized	Unfertilized	Fertilized
57	100	100	100	100
48	97	73	111	87
41	108	70	106	89
36	104	61	102	82
33	84	48	101	63
25	91	41	105	54

Table 3. *The effect of diurnal light period and of fertilizing on the dry matter content (%) of the shoots.*

Light period	Exp. 14.2.—19.3.		Exp. 3.4.—28.4.	
	Unfertilized	Fertilized	Unfertilized	Fertilized
Short	13.61	10.81	11.05	10.24
Long	16.93	10.93	12.40	10.87

erence has continued to grow with the prolongation of the diurnal period of light. The positive effect of the intensity of light, too, has appeared more distinctly in the fertilized than in the unfertilized wheat (Table 6).

The dry matter yield of the roots, on the other hand, has been much greater in the unfertilized wheat. The effect of the lengthening of the diurnal period of light (Table 5), and of the intensity of light (Table 6) on the root yield of wheat has been positive.

The total dry matter yield has been increased very much by the fertilizing. The effect of the length of the light period (Table 5) and of the intensity of light (Table 6) has manifested itself more in the fertilized than in the unfertilized wheat. When the intensity of light has been poorest (25 per cent), the effect of fertilizing on the total dry matter yield has been but slight (Table 7).

The dry matter carbon content (Table 8) has been negatively affected by the fertilizing; however, on an average fertilizing has increased the *carbon yield*. In the experiments carried out in February—March fertilizing increased the carbon yield about 25 per cent only, when the intensity of light has been at its lowest (25 per cent). Taking it that the dry matter carbon content also varied in this way in the experiments carried out in April, as is shown in Table 8, fertilizing did not increase the carbon yield at all when the intensity of light was only 25 per cent (cf. Table 7). In the highest (57 per cent) light intensity, however, fertilizing has very considerably increased the carbon yield of wheat. The positive effect of fertilizing on the carbon yield (Table 8) as well as on the dry matter yield (Table 5) of the wheat has been decisively dependent on its increasing the shoot yield. Thus, fertilizing has increased the dry matter and carbon yield partly »at the expense of the root system».

In different light intensity and fertilizing conditions the variation in the carbon content has been parallel to the variation of the hydrogen content; both in shoots and in roots the H : C ratio has been stable, 0.14. On the other hand the ash: carbon ratio has been different in various growth conditions of wheat.

Table 4. *The effect of intensity of light and of fertilizing on the dry matter content (%) of the shoots.*

Intensity of light (%)	Exp. 14.2.—19.3.		Exp. 3.4.—28.4.	
	Unfertilized	Fertilized	Unfertilized	Fertilized
57	19.76	12.90	14.45	12.15
48	17.09	11.40	12.21	11.46
41	15.29	11.11	12.28	10.70
36	14.65	10.36	11.43	9.94
33	13.36	9.72	10.50	9.71
25	11.50	9.74	9.46	9.49

Table 5. *The effect of diurnal light period and of fertilizing on the dry matter yield (g per 30 plants).*

Light period	Exp. 14.2.—19.3.		Exp. 3.4.—28.4.	
	Unfertilized	Fertilized	Unfertilized	Fertilized
A. Shoots				
Short	1.58	3.12	1.13	1.77
Long	1.68	3.98	1.55	2.58
B. Roots				
Short	1.06	0.74	0.97	0.73
Long	1.06	0.88	1.24	0.93
C. Shoots + roots				
Short	2.64	3.86	2.10	2.50
Long	2.74	4.86	2.79	3.51

Table 6. *The effect of intensity of light and of fertilizing on the amount of the dry matter yield.*

Intensity of light (%)	Exp. 14.2.—19.3.		Exp. 3.4.—28.4.	
	Unfertilized	Fertilized	Unfertilized	Fertilized
A. Shoots				
57	100	100	100	100
48	82	64	94	81
41	78	60	89	78
36	74	49	80	67
33	55	36	73	50
25	52	31	69	42
B. Roots				
57	100	100	100	100
48	79	63	104	79
41	63	63	79	70
36	52	49	63	54
33	41	36	51	44
25	34	33	41	35
C. Shoots + roots				
57	100	100	100	100
48	81	64	99	80
41	71	61	83	75
36	64	49	72	62
33	48	36	62	48
25	44	31	55	40

Table 7. *The effect of fertilizing on the amount of the dry matter yield by varying intensity of light.*

Intensity of light (°/o)	Amount of dry matter yield of unfertilized wheat (fertilized = 100)					
	Exp. 14.2.—19.3.			Exp. 3.4.—28.4.		
	Shoots	Roots	Shoots + roots	Shoots	Roots	Shoots + roots
57	36	123	52	51	114	70
48	45	149	64	58	155	86
41	45	123	60	59	128	78
36	55	129	68	61	135	80
33	55	140	70	75	137	91
25	60	130	73	84	141	98

Table 8. *The effect of diurnal light period, intensity of light, and fertilizing on the carbon yield of the wheat (exp. in February—March).*

Quantity recorded	Fertilizing	Intensity of light		Light period	
		57 °/o	25 °/o	Short	Long
A. Shoots					
Dry matter (g)	not fertilized	2.22	1.16	1.61	1.78
» »	fertilized	6.23	1.93	3.90	4.26
Carbon (°/o)	not fertilized	47.31	44.74	45.48	46.58
»	fertilized	44.45	41.69	43.01	43.13
Carbon (g)	not fertilized	1.05	0.52	0.74	0.83
»	fertilized	2.77	0.80	1.71	1.86
B. Roots					
Dry matter (g)	not fertilized	1.73	0.59	1.07	1.25
» »	fertilized	1.41	0.45	0.91	0.96
Carbon (°/o)	not fertilized	49.95	48.91	51.33	47.54
»	fertilized	45.19	47.63	46.31	46.52
Carbon (g)	not fertilized	0.86	0.29	0.55	0.42
»	fertilized	0.64	0.21	0.43	0.43
C. Shoots + roots					
Dry matter (g)	not fertilized	3.95	1.75	2.68	3.03
» »	fertilized	7.64	2.39	4.81	5.22
Carbon (°/o)	not fertilized	48.35	46.29	47.44	47.03
»	fertilized	44.63	42.44	43.65	43.82
Carbon (g)	not fertilized	1.91	0.81	1.29	1.25
»	fertilized	3.41	1.01	2.14	2.29

Table 9. *The effect of diurnal light period and of fertilizing on the percentage of total dry matter yield represented by the roots of wheat.*

Light period	Exp. 14.2.—19.3.		Exp. 3.4.—28.4.	
	Unfertilized	Fertilized	Unfertilized	Fertilized
Short	39.0	19.8	44.2	29.2
Long	37.9	17.7	43.6	25.7

Table 10. *The effect of intensity of light and of fertilizing on the percentage of total dry matter yield represented by the roots of wheat.*

Intensity of light (‰)	Exp. 14.2.—19.3.		Exp. 3.4.—28.4.	
	Unfertilized	Fertilized	Unfertilized	Fertilized
57	43.8	18.5	49.0	29.8
48	42.2	18.2	51.6	29.7
41	38.7	19.4	44.6	27.5
36	35.5	18.5	42.4	25.7
33	36.9	18.9	39.5	26.7
25	33.8	19.1	36.2	25.4

	Shoots	Roots
Unfertilized	0.27	0.29
Fertilized	0.39	0.38
Intensity of light 57 ‰	0.28	0.31
Intensity of light 25 ‰	0.38	0.36

Fertilizing and weakening intensity of light, which have reduced the dry matter carbon content (Table 8), have considerably increased the ash content:

	Shoots	Roots
Unfertilized	12.32 ‰	14.13 ‰
Fertilized	16.46 ‰	17.57 ‰
Intensity of light 57 ‰	12.62 ‰	14.60 ‰
Intensity of light 25 ‰	16.16 ‰	17.10 ‰

The root-top ratio has been considerably higher in the unfertilized than in the fertilized wheat. The lengthening of the diurnal light period has reduced the root-top ratio of fertilized wheat (Table 9). On the other hand, the intensity of light has had almost no effect on the root-top ratio in the fertilized wheat, although in the unfertilized wheat the positive effect of light intensity on the root-top ratio has been considerable (Table 10).

Discussion

Lundegårdh (4, p. 461) found in his investigations that increased CO₂ content of the atmosphere, in compliance with the law of relativity, raised the yield of fertilized sugar-beet more than that of unfertilized. Similarly, in our experiments, fertilizing increased the positive effect of light intensity on the dry matter yield of wheat (Table 6). As, moreover, in the lowest light intensity studied (25 per cent), fertilizing increased the carbon yield of wheat very little or not at all (Tables 7 and 8), it may be concluded that in such poor light intensity the fertilizing, although it definitely deepened the green colour of the foliage, did not, to any extent worth mentioning, increase the

ability of the wheat to utilize light energy. On the other hand, in a light intensity of 57 per cent (which obviously represents less than half the daily light dose during the growth period in the summer) the effect of fertilizing on the carbon yield of wheat was very perceptible.

The fact that light intensity had a more marked effect on the dry matter yield than the green yield of wheat (Tables 2 and 6) can be understood from the fact that the dry matter of plants is mainly a result of CO_2 assimilation (cf. Table 8). However, a fraction only of the green yield consisted of dry matter (Table 3), and consequently the variations in water content (Table 4) affected the amount of the green yield considerably. Although, in unfertilized wheat, the dry matter yield of shoots, with increasing light intensity, revealed an average increase of 1.65 times (Table 6), the amount of green yield did not increase at all (Table 2) as the water content was simultaneously reduced (Table 4). As, furthermore, the absolute yield of water was also somewhat reduced with light intensity increasing (cf. Tables 2 and 4), although no differences in temperature were connected with the light intensity variations (sunny days were few and the outdoor temperature remained below 0°C , for which reason proximity to the window at least did not increase the temperature and the resulting transpiration), the idea suggests itself that *increased intensity of light has reduced the ability of the plants to retain water*. This opinion is supported by the fact that abundant fertilizing, which has obviously increased the ability of the shoots to retain water (Table 3), has reduced the part played by light intensity in the variation of the water content of the shoots (Table 4).

The negative effect of the intensity of light on the ability of wheat shoots to retain water can be understood if, according to Lundegårdh (1950, p. 198), the effect of light on the opening of the stomata is understood as a function of the intensity of assimilation. In the present experiments variations in light intensity have affected the assimilation very considerably (Table 8). Hence it is to be expected that increased light intensity has increased the opening of the stomata, reducing accordingly the ability of the plant to retain water. In compliance with this opinion is the fact that abundant fertilizing (of which the increasing effect on the water-retaining ability of wheat apparently depends mainly on factors other than the heightened capacity of the plant surface to prevent transpiration) has reduced the effect of light intensity on the dry matter content of wheat shoots (Table 4). In addition, the prolongation of the diurnal period of light, which in the experiments effected in April was not connected with any rise in temperature whatever, increased the dry matter content of unfertilized wheat more than that of fertilized wheat (Table 3).

Like the dry matter content (Tables 3 and 4), the root-top ratio (Tables 9 and 10) was lower in fertilized wheat than in unfertilized. Furthermore, both decreased with reduced light intensity, in unfertilized wheat more sharply than fertilized. Thus it seems that the root-top ratio, in part at least, depends on the same factors as the dry matter content. It is natural that when transpiration is abundant and the water content of the shoots remains low, the proportion of the roots increases compared with the shoots, i.e. the transpiratory parts of the plants. As high light intensity is accompanied by abundant transpiration and low water content, even when no increase in temperature is connected with increase in light intensity, it is evident that the root-top ratio grows with increasing light intensity (cf. also 5 and 6). It is true that the increase in light intensity has a positive effect on the development of the cereals (5, 6), which again has a negative effect on the root-top ratio (7), but in the present experiments the positive effect of increased transpiration (lower water content) on the root-top ratio has apparently been of greater importance. However, with very abundant fertilizing, the internal water-retaining ability of the tissues has evidently been so great that the significance of stomatal transpiration has been of secondary importance and the increase in root-top ratio with increased light intensity has remained minute (Table 10). Thus the high root-top ratio and the positive energy economy of wheat have been partly parallel phenomena, the latter reacting according to the intensity of light, the former according to the resulting intensity of transpiration.

On the other hand prolongation of the diurnal period of light reduces the root-top ratio of the long-day plants more if they have grown in intense illumination than if the light has been weak (5). It is generally known, too, that photoperiodical irritation, the accelerating effect on development, and the resulting reducing effect on root-top ratio of the long photoperiod manifest themselves more markedly the higher the light intensity at noon. With sufficient daylight intensity the effect of the long photoperiod on the rate of development of the plants is so considerable that it exceeds the effect of light on the opening of the stomata when the effect of these two factors on the root-top ratio is in question. Since fertilizing has increased the water-retaining ability of the shoots, the accelerating effect of the photoperiod on development and on the root-top ratio has manifested itself more markedly in the fertilized wheat than in the unfertilized (Table 9). Thus, the degree of reducing-effect of the photoperiod on the root-top ratio depends both on the energy balance and, to some extent, also on the fertilizing of the long-day plant.

Conclusions

The following are the main conclusions to be drawn from the results of the present investigations:

1. Though the intensity of light was even less than half the normal light intensity during the summer growth period fertilizing had a very positive effect on the yield of wheat.

2. However, when light intensity decreased further until it was less than half that in the previous paragraph, fertilizing lost practically all effect.

3. Fertilizing increased the shoot yield of wheat partly »at the expense of the root system».

4. Decreases in light intensity reduced the root-top ratio of wheat and the dry matter content of its shoots. This was probably caused, partially at least, by the closing of the stomata.

5. The degree of the reducing-effect of the long photoperiod on the root-top ratio of long-day cereals depends both on the energy balance and to some extent also on the fertilizing of the plant.

References

1. Bukatsch, Fr.: Über den Einfluss verschiedener mineralischer Ernährung auf den Blatt-pigmenthalt II. — *Phyton, Annales rei Botanicae* 3: 173. 1951.
2. Kolisko, L.: Wirkung von Licht und Finsternis auf das Pflanzenwachstum. — *Philosophisch-Antroposophischer Verlag am Goetheanum Dornach Schweiz*, p. 113. 1926.
3. Liesegang, H. & Remy, Th.: Untersuchungen über die Rückwirkungen der Kaliversorgung auf Chlorophyllgehalt, Assimilationsleistung, Wachstum und Ertrag der Kartoffeln. *Acker- und Pflanzenbau, Landwirtsch. Forsch.* 1926: 31.
4. Lundegårdh, H.: *Lärobok i växtfysiologi med växtanatomi*. — Stockholm 1950.
5. Pohjakallio, O.: On the effect of the intensity of light and length of day on the energy economy of certain cultivated plants. — *Acta Agricult. Scand.* 1: 153. 1951.
6. — Kauran nokitaudin ilmenemisestä erilaisissa valoisuussuhteissa (Über das Auftreten von Brand bei Hafer unter verschiedenen Belichtungsverhältnissen). — *J. Scient. Agricult. Soc. of Finland* 24 (1952): 24. 1951.
7. Roberts, R. H. & Struckmeyer, B. E.: Anatomical and histological changes in relation to vernalization and photoperiodism. — *Vernalization and Photoperiodism* ed. by A. E. Murneek and R. O. Whyte, p. 91. Waltham, 1948.

Metal-Protein Complexes in the Potato

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The role of the trace elements in plants is still little understood, though it has been studied from the nutritional and to some extent the enzymatic point of view. By the former method, Fe, Cu, Zn, Mn, B, and Mo have been found essential for the growth and development of plants (9); by the second method Fe, Cu, and Zn have been shown to act as prosthetic groups (or constituents of them) in several enzymes, e.g. Fe in cytochromes, cytochrome oxidase, peroxidase and catalase, Cu in tyrosinase and ascorbic acid oxidase, Zn in carbonic anhydrase (2). On the other hand, in spite of the many efforts to find enzymes that contain Mn and B, there is no good evidence that they ever form fixed prosthetic groups. Bertrand et al (1), in opposition to most other workers, have concluded that Mn is the prosthetic group of laccase, but this has been disproved by Tissieres (11). Mn in the free ionic form is known to activate some enzymes but in such cases it is not attached to the protein (10).

The purpose of the present investigation is to follow up these important biochemical discoveries, and attempt to learn more about the physiologic relations between the trace elements and the proteins. There are many questions to be answered. Are these trace elements essential solely because of their association with the proteins or do they also have some other role? Are there constant amounts of metal-protein complexes in the plant or are these formed in larger quantity when for instance, growth commences? Do the complexes occur in the granules of the protoplasm or as free proteins or both? These and many other questions must be answered before we can have an adequate picture of the role of the trace elements.

Methods

The isolation and preliminary fractionation of potato tuber proteins has already been described (3). Burbank Russet potatoes were again used in this investigation. Some minor differences in technique were adopted. When pH of extraction and precipitation was to be varied, this was accomplished by using K_2HPO_4 or KH_2PO_4 in quantities determined by trial and error. The amount of NH_4OH to be added to the precipitating $(NH_4)_2SO_4$ was similarly determined.

If the pH of extraction and precipitation was below 6.5 some of the dialyzed materials precipitated during dialysis. Thus a rough, incomplete separation into »soluble» and »insoluble» fractions could be obtained simply by centrifuging for 2 minutes at 600 XG. Though this usually failed to cause any separation at pH 7.0—8.0 (except after several months' storage of the potato powder, e.g. in experiment 1 table 2), it caused an increasing yield of »insoluble» fraction with decreasing pH and an increasing clarity of the supernatant until this became perfectly clear at pH's of 5.5 to 5.0. At the lower pH's it was possible to recover all the insoluble fraction by eliminating the celite and filtering through Whatman no. 50 or S and S no 576. The insoluble fraction could now be obtained by scraping it off the filter paper.

In the first set of experiments, colorimetric methods of analysis were used for the trace elements Fe, Zn, and Mn according to Parks et al (6), Cu according to Sherman and McHargue (8). The rest of the analyses were performed spectrographically (7). The spectrographic method has the advantage of enabling the use of much smaller amounts of material (a 40—50 mg. sample served for all three elements tested) and of being more sensitive to Mn than the colorimetric method. Each value was an average of two determinations. The spectrographic method in the earlier experiments (Table 3) was subject to an error of as large as 20 per cent. In all later experiments the maximum error was usually below 10 per cent. However, other sources of error added to the variation and only relatively large and consistent differences can be relied on.

The procedure was as follows. About 50 mg. of each protein fraction was digested in a 10 ml. beaker with 0.5 ml. redistilled HNO_3 until almost dry. 0.3 ml. 50 per cent redistilled HNO_3 and 0.3 ml. 60 per cent perchloric acid were then added and the sample was again digested till practically dry. Since perchloric acid was found to suppress the spectrum lines of the elements tested for (7), the beakers were then transferred to an oven and kept at $500^\circ C$ for two hours. After cooling, the ash was dissolved in 0.25 ml. redistilled 15 per cent HCl and warmed. The ash solution was then transferred to a small test-tube graduated at 0.5 ml. and the beaker rinsed with more HCl to make up to the 0.5 ml. mark.

Graphite electrodes (5/16 inch diameter) were cut into 2-inch lengths and a crater

was ground in one end. Smaller size graphite (3/16 inch diameter) for use as the cathode was also cut into 2-inch lengths. All electrodes were then pre-arc'd for 30 seconds and then dipped into a solution of 10 per cent paraffin in toluene and allowed to dry under a heat lamp.

A micropipette designed and built by Dr. E. E. Pickett was used throughout the work. 0.10 ml. of the internal standard (0.5 g/l of NiCl_2) was added to the electrodes and dried. This was followed by the addition of 0.10 ml. of a solution containing 5.882 g. of KCl, 0.588 g. of NaCl, 2.941 g. of CaCl_2 , and 0.588 g. of MgCl_2 per liter. These elements were added for a stabilizing or buffer effect since there were fairly large amounts of these elements present in the samples and the amount varied from one sample to the next. Without the buffer these major elements would exert an extraneous effect on the elements being determined. After the buffer solution had dried, 0.10 ml. of the sample was likewise placed on the electrode and dried. During the operations of filling and drying the electrodes, precautions were taken to prevent the entrance of dust or other foreign material into the samples. All the liquids were dried on the electrodes with a heat lamp.

Exposures of these samples were made in the following way: A 21 foot Jarrell-Ash grating emission spectograph of the Wadsworth type with a plate factor of 3.5 Å/mm was used with a slit length of four and a width of 50 mμ. No lens was employed and a half sector having a ratio of 10 : 1 was placed immediately in front of the slit so that copper could be exposed simultaneously with the other elements. The electrode containing the sample was burned as the anode in a D.C. arc of 7 amps and 280 volts impressed. The exposures were made for 30 seconds on SA No. 1 film which was developed for 4 minutes in D-19 developer. The film was read on a Jarrell-Ash Comparator Microdensitometer. The actual amounts of the metals iron, copper and manganese were then determined from previously prepared working curves, second order spectra being used.

The best working curves were obtained with the addition method whereby known amounts of the desired elements were added to a typical protein sample (5). By extrapolation the amount of element present in the original sample could be determined and standard curves for each element were constructed. This method has the advantage that the working curves are less artificial since they are prepared from standards having approximately the same elemental composition as the samples to be measured. The spectrum lines used were iron 2788.1 Å, manganese 2801.1 Å, copper 3274.0 Å, nickel 2746.7 Å, and nickel 3225.0 Å.

Each value in the tables is the average of two determinations which usually agreed within less than 10 per cent.

The two methods can only be roughly compared since they were used in different years, at different times of the year, and on different lots of potatoes. Mn values were of the same order in the two sets of results; Cu and Fe values were in some cases considerably higher in the colorimetric determinations.

Results

1. Distribution of trace elements in the potato tuber.

The object of this experiment was to determine whether or not the trace elements are concentrated in the proteins. Earlier attempts (using Truog's

Table 1. *Fe, Cu, and Zn contents of potato powder and of crude protein extracted from it. Determined colorimetrically. Each value an average of 2—4 determinations.*

Element	Quantity of element $\mu\text{g/g}$		Portion in crude protein (% of total in potato)
	in potato powder	in crude protein	
Fe	42	567	27
Cu	13	145	23
Zn	5.5	72	26

quinalizarine method) failed to detect any B in the crude protein though detectable amounts occurred in the potato as a whole (about 20—30 $\mu\text{g/g}$ dry matter). Consequently, the analyses below were confined to Fe, Cu, Zn, and Mn. The proteins were extracted and precipitated at about pH 7.5.

Table 1 shows that Fe, Cu, and Zn were present in the potato as a whole and also in the crude protein. In each case the quantity per gram protein was about $12\times$ that per gram potato powder. Yet since the protein comprised only 2 per cent of the dry matter of these potatoes this meant that 75 per cent of these trace elements was not associated with the proteins. Mn was detected only once in the crude protein and it was present in the potato powder in amounts just sufficient to be detected colorimetrically (5—10 $\mu\text{g/g}$). Later spectrographic analyses succeeded in determining Mn in the proteins, usually in amounts too low to be measured colorimetrically (see below).

2. Effect of pH of extraction and precipitation on trace element content of protein.

One obvious objection to the above results is that the trace elements may be simply adsorbed on the proteins rather than chemically combined. This may seem especially likely in view of the rather high pH (7.5) employed above. The soluble inorganic trace elements might conceivably be precipitated onto the proteins. When, however, the method used is considered, this seems unlikely. The proteins comprise only 2 per cent of the dry matter and are associated with 25 per cent of the trace elements. Any precipitate formed during extraction is likely to be mainly adsorbed by the other 98 per cent, particularly since most of this is finely ground, insoluble material whereas the proteins go into solution. During protein precipitation by $(\text{NH}_4)_2\text{SO}_4$, there would be no further precipitation of trace elements since the pH is constant.

In order to test this point experimentally, extraction and precipitation of proteins was performed at various pH's from about 5.0 to 8.0, and trace elements determined. If they occurred in the proteins due to adsorption, the amount present would rise with the pH, not only because the solubility of

Table 2. *Quantities of crude protein obtained at different pH's (g per 10 g potato powder).*

pH range	Experiment No.			Average
	1	2	3	
7.4—7.8	0.1586	0.1814	0.1484	0.1628
7.0—7.3	0.1418	0.1638	0.1559	0.1535
6.5—6.7	0.1488	0.1562	0.1295	0.1448
5.7—6.1	0.1514	0.1520	0.1221	0.1452
4.8—5.4	0.1330		0.1145	0.1238

the trace elements decreases with rising pH, but also because the negative charge on the proteins increases.

The quantity of crude protein obtained decreased rather regularly with the pH (table 2). The proteins also were less stable at the lower pH's as stated above. One might therefore suspect some denaturation and perhaps even splitting off of combined trace elements.

Table 3 shows the results obtained. The pH has little if any effect on the copper content of the extracted proteins. A slight increase appears to occur with decreasing pH, which is just the opposite of what would be expected due to adsorption. However, this gradation is partially nullified when total protein copper is calculated, since there is a decrease in protein extracted with decrease in pH (table 2). The differences then fall within the limits of error of the method used.

In the case of Mn, there is a definite maximum at a pH 7.1 to 7.3 with a marked drop on both sides. The sharp increase from pH 6.5 to 7.1 might

Table 3. *Effect of pH of extraction and precipitation on trace element content of crude protein ($\mu\text{g/g}$ protein).*

pH	Series 1	Series 2	Series 3	Average
a) Cu				
7.4—7.7	24	42	17	24
7.1—7.3	26	38	17	27
6.5—6.6	29	51	10	30
5.8—6.1	38	42	19	33
4.8—5.4	36		20	33
b) Mn				
7.4—7.7	14	1	2.5	5.8
7.1—7.3	23	15	4.0	14.0
6.5—6.6	10	10	3.5	7.8
5.8—6.1	10	7	1.5	6.2
4.8—5.4	11		1.5	6.8

Table 4. *Metal content of the crude protein fraction of potato tubers ($\mu\text{g/g}$ of protein). 10 days at the given temperature.*

Date	Iron		Copper		Manganese	
	2° C.	25° C.	2° C.	25° C.	2° C.	25° C.
2/1/50	220	230	33	33	4.9	5.4
5/5/50	260	320	31	32	6.1	6.2
7/27/50	160	210	21	28	3.1	5.2
8/12/50	250	250	16	17	4.7	5.6
New Tubers						
9/1/50	340	300	35	27	4.6	4.2
10/1/50	300	410	23	25	4.2	5.8
11/1/50	290	370	26	32	3.9	4.9
12/13/50	330	320	34	25	5.8	6.8
1/1/51	340	360	24	37	5.7	5.8
2/1/51	390	380	19	27	5.5	6.0
3/1/51	370	470	24	22	5.7	6.2
4/1/51	510	460	24	26	7.1	7.9
5/1/51	390	480	16	18	10.8	7.4
6/1/51	470	660	85	68	7.1	10.8
7/1/51	510	370	47	25	10.3	6.1

be explainable on the basis of adsorption, but the similar sharp drop on the alkaline side is not.

Preliminary results with Fe were, in general, the same, but the error of the method was at this time too great and the results are not presented. It seems safe, however, to conclude that the three trace elements tested are normal constituents of the proteins and are not simply precipitated on them during extraction. This result is to be expected in the case of Fe and Cu and possibly Zn, since these have all been found to act as prosthetic groups of enzymes. In the case of Mn the results are perhaps more interesting since this element has not been shown to act as a firmly held prosthetic group. However, the quantities of Mn present are so low that further enrichment by fractionation will be necessary to prove its association with the protein.

The importance of impurities was tested by using dithizone extracted and recrystallized $(\text{NH}_4)_2\text{SO}_4$. Since this substance is added in largest amount, it is likely to be the greatest source of contamination. In table 3 above, experiments 1 and 2 were performed with unpurified $(\text{NH}_4)_2\text{SO}_4$, experiment 3 with purified $(\text{NH}_4)_2\text{SO}_4$. Spectrographic analyses showed traces of Fe, Cu, and Mn in both the purified and unpurified salt, though definitely less in the latter.

The smaller quantities of Cu, and Mn in this series may perhaps be due to the use of the purer salt, though one would not expect the dithizone method to affect the Mn content of the salt as markedly as the Cu content.

Table 5. *Metal content of the albumin protein fraction of potato tubers ($\mu\text{g/g}$ of protein). 10 days at the given temperature.*

Date	Iron		Copper		Manganese	
	2° C.	25° C.	2° C.	25° C.	2° C.	25° C.
2/1/50	260	170	125	66	5.2	8.2
5/5/50	250	250	71	47	5.0	5.2
7/27/50	220	170	45	35	4.5	4.2
8/12/50	310		30		4.6	
New Tubers						
9/1/50	350	360	45	42	4.1	5.1
10/1/50	300	250	43	45	4.5	4.7
11/1/50	350	340	44	37	4.6	4.5
12/13/50	290	160	32	27	3.6	3.6
1/1/51	570	630	80	87	6.6	8.3
2/1/51	560	500	55	39	7.9	6.8
3/1/51	480	450	37	41	6.2	5.9
4/1/51	460	500	47	43	8.4	7.3
5/1/51	460	390	29	23	7.2	7.3
6/1/51		440		65	8.6	6.9
7/1/51	400	320	73	35	5.9	6.4

Table 6. *Metal content of the acid insoluble protein fraction of potato tubers ($\mu\text{g/g}$ of protein). 10 days at the given temperature.*

Date	Iron		Copper		Manganese	
	2° C.	25° C.	2° C.	25° C.	2° C.	25° C.
2/1/50	240	—	67	—	2.7	—
5/5/50	375	340	70	47	1.2	2.1
7/27/50	255	215	53	37	1.3	1.1
8/12/50	400	—	40	—	1.0	—
New Tubers						
9/1/50	380	360	57	53	1.7	2.0
10/1/50	460	310	70	55	1.6	0.3
11/1/50	330	340	55	59	1.3	0.3
12/13/50	360	270	44	49		1.2
1/1/51	600	900	98	270	1.6	1.0
2/1/51	445	390	71	74	0.5	1.0
3/1/51	510	495	68	74	1.9	2.5
4/1/51	440	525	59	73	2.1	3.1
5/1/51	460	575	41	45	1.7	4.0
6/1/51					2.4	3.2
7/1/51		475		81	3.5	5.4

At any rate, the effect of pH on metal content of the protein is the same in all three cases.

It seemed advisable to use a lower pH than 7.5 for future extractions, partly because of the above evidence of maxima for trace elements at pH's 6.5—7.0, partly because it is approximately the pH of protoplasm.

Table 7. *Metal content of the acid soluble albumin protein fraction of potato tubers* ($\mu\text{g/g}$ of protein). 10 days at the given temperature.

Date	Iron		Copper		Manganese	
	2° C.	25° C.	2° C.	25° C.	2° C.	25° C.
2/1/50	28	330	68	97	4.6	4.8
5/5/50	86	110	60	63	4.9	7.9
7/27/50	29	180	49	37	3.5	3.9
8/12/50	58	—	35	—	5.6	—
New Tubers						
9/1/50	28	46	60	64	7.5	8.5
10/1/50	90	19	57	62	5.9	4.4
11/1/50	34	43	55	47	5.8	5.4
12/13/50	16	10	43	48	3.6	3.7
1/1/51	130	95	90	110	3.9	6.8
2/1/51	0	22	98	67	6.4	6.8
3/1/51	78	63	56	54	6.3	5.6
4/1/51	120	105	81	75	8.3	8.3
5/1/51	58	64	57	71	5.5	4.0
6/1/51	—	65	—	140	—	4.5
7/1/51	50	61	—	88	—	9.0

3. Seasonal determinations of trace elements in proteins of dormant and active tubers.

The attempt was next made to determine whether or not there were any changes in quantity of each trace element associated with the crude protein either during storage at $+2^{\circ}\text{C}$ or on transfer to $+25^{\circ}\text{C}$ for 10 days. Table 4 fails to reveal any significant correlations. The variations from sample to sample are large, though the duplicates nearly always checked to within 10 per cent.

The metal content of the albumin fraction was also determined, and again no significant correlations were observable either with storage period or with change over from the dormant to the active state (Table 5). The Cu content of the albumins was in almost every case higher than that of the crude. When this albumin fraction was split into an acid-insoluble (i.e. insoluble at pH 5.3) and an acid-soluble fraction, certain interesting relationships appeared. Most of the albumin iron (75—95 per cent) was always found in the acid-insoluble fraction, most of the albumin manganese (70—95 per cent) in the acid-soluble fraction (Tables 6 and 7). The copper was equally distributed between the two. During the winter and summer of 1950, the iron in the acid-soluble protein fraction was always much higher in tuber halves kept at $+25^{\circ}\text{C}$ for 10 days. But this result was not obtained in the 1950—51 tubers.

Discussion

Though Fe, Cu, Zn, and Mn are much more concentrated in the proteins than in the potato as a whole, about 75 per cent of these elements was not associated with the proteins. This value is, of course, not necessarily true of all potato tubers. It is not possible, from these results, to preclude some secondary role for these elements besides that associated with the metal-protein complexes. On the other hand, the non-protein component may be the result of luxury consumption of these elements, or it may serve as a reserve for the synthesis of new metal-protein complex when the tuber sprouts. In the case of Mn, the concentration in the crude protein is much lower than that of the above three elements. It remains to be proved, whether a Mn-protein complex also exists in the potato though in much smaller quantity than the Fe, Cu, and Zn protein complexes.

It should first be pointed out that in spite of the good agreement between duplicate spectrographic determinations, all the values for the metal content of proteins and protein fractions can only be considered as semi-quantitative. This is partly due to the loss of metal during dialysis. Thus, the iron and manganese content of the acid-insoluble and the acid-soluble albumins always added up to far less than the content of the mixed parent albumin. Long and Levitt (4) have shown that definite losses do occur during dialysis. The copper contents of the two daughter fractions, on the other hand, added up to more than the parent fraction. This is undoubtedly due to capture of copper from the distilled water that was used to dialyze the fractions, for though the distilled water gave little or no detectable dithizone test, proteins can take up copper from very weak solutions.

Since, however, the determinations were made under similar conditions, the relative values should be dependable. Thus, there seems no question that the iron content of the crude protein is of the order of $10 \times$ the copper content and $100 \times$ the manganese content. There can also be no doubt but that the iron content of the acid-insoluble albumins is much higher than that of the acid-soluble albumins whereas the relative manganese contents are the reverse of this. It has been shown (12) that the catalase activity of the albumins is probably confined to this acid-insoluble fraction. Similarly, the iron content of the 25° C acid-soluble albumin was higher during one year than the content of the similar 2° C fraction. Again, peroxidase values were found to be associated with this difference (12). Thus, it is felt that these protein-metal values are at least of use in pointing to possible enzyme relationships among the dozens or scores that might otherwise be suspected.

Summary

(1) Fe, Cu, Zn, and Mn (but not B) were found in the isolated proteins of potato tubers.

(2) Fe, Cu, and Zn were present in the proteins to the extent of about $12 \times$ their concentrations in the potato as a whole. Since the proteins comprised about 2 per cent of the dry matter, this meant that about 25 per cent of the total Fe, Cu, and Zn in the tuber was associated with the proteins. Mn was less concentrated in the proteins than in the tuber as a whole.

(3) The solubility of the proteins decreased with pH of extraction and precipitation (from 8 to 5) as did the total crude protein extracted.

(4) In spite of the above changes produced by changes in pH of extraction and precipitation, the Cu content per unit total crude protein extracted varied little with the pH, Mn showed a maximum at pH 7.1—7.3, but no significant differences at the other pHs. Such variations as occurred were not in the direction to be expected from adsorption. This is taken to mean that these elements are, in the main, normally associated with the proteins rather than being precipitated onto them during extraction.

(5) Fe, Cu, Zn, and Mn were all present in both the soluble and the insoluble protein fractions. The Fe content of the crude protein was about $10 \times$ the Cu content, $100 \times$ the Mn content. The Cu content of the albumins was higher than that of the crude protein. The acid-insoluble albumins contained 75—95 per cent of the albumin Fe, but only 5—25 per cent of the albumin Mn. No significant differences in distribution of Fe, Cu, or Mn in the proteins could be detected either associated with length of storage or with transition from dormancy to growth.

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References

1. Bertrand, D., Belval, H. & Legrand, G.: Études sur la laccase. — Bull. Soc. Chem. Biol. 29: 607. 1947.
2. Green, D. E.: Mechanisms of biological oxidations. — 1940.
3. Levitt, J.: The isolation and preliminary fractionation of proteins from dormant and growing potato tubers. — Plant Physiology 26: 59. 1951.
4. Long, W. G. & Levitt, J. (In press).
5. Nelson, R. C., Hamm, P. C. & Tsiang, Y. S.: Spectrographic analysis of plant ash for several elements simultaneously. — Plant Physiol. 18: 699. 1943.
6. Parks, R. Q., Hood, S. L., Hurwitz, Ch. & Ellis, G. H.: Quantitative chemical micro-

- determination of twelve elements in plant tissue. — *Ind. and Eng. Chem. (Anal. Ed.)* 15: 527. 1943.
7. Pickett, E. E. & Todd, G. W.: Suppression of line intensities by perchloric acid. — *Journal of the Optical Society of America* 41: 361. 1951.
 8. Sherman, G. D. & McHargue, J. S.: Methods for determination of copper and zinc in soil. — *Assoc. Off. Ag. Chem. Journ.* 25: 510. 1942.
 9. Stiles, Walter: Trace elements in plants and animals. 1946.
 10. Sumner, James B. & Somers, G. F.: Chemistry and methods of enzymes. 1947.
 11. Tissières, A.: Constitution and properties of laccase. — *Nature* 163: 480. 1949.
 12. Todd, G. W. (In press).

2,4-Dichloranisol—Auxin Interactions in Root-Growth

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Introduction

In the last decade or so a vast new field has opened up in the study of the biochemistry of growth as the result of discoveries of the marked antagonisms that can arise in some cell systems between metabolites and their related structural homologues. The antagonisms between the sulphonamides and para-aminobenzoic acid and also between the vitamins and the vitamers are now the classic examples of this phenomenon. Physiologists interested in the mechanism of action of the auxins have also exploited this line of attack with promising success in some cases. Compounds which have been claimed to »antagonise» auxin action in one or more of its aspects are 2,4-dichloranisol (Bonner, 8), coumarin (Veldstra and Havinga, 23), certain isobutyric acid homologues of auxins (Burström, 13), 2,3,5-tri-iodobenzoic acid (Galston, 16), α -(2-naphthylmethylsulphide)-propionic acid (Åberg, 1), laevo- α -(2-naphthoxy)propionic acid (Åberg, 2; Wain, 24), maleic hydrazide (Leopold and Klein, 17) and trans-cinnamic acid (Overbeek et al., 22).

Some of these compounds (i.e. 2,4-dichloranisol, tri-iodobenzoic acid), having been accepted, in the view of the authors rather uncritically, as established »anti-auxins», are being used as tools to investigate some of the problems of auxin physiology. For example, the augmentation of flower production in *Xanthium* by application of 2,4-dichloranisol (DCA) (Bonner, 8; Bonner and Thurlow, 10), in barley by coumarin (Leopold and Thimann, 18), and in tomatoes by tri-iodobenzoic acid (Galston, 16) is now regarded as strong evidence that flowering is correlated with a low level of effective

auxin in the plant, having in these cases been induced by the antagonistic action of the applied »anti-auxin«. But before such far-reaching conclusions are drawn, we should make quite certain that a true auxin antagonism can be unequivocally established from observations on growth (preferably extension growth) of single plant organs or homogeneous growing tissue excised from them. Experiments along these lines were started by the senior author (L.J.A.) in 1948 on the growth of radicles of cress seedlings and 2,4-dichloroanisole (subsequently to be called DCA) was one of the compounds investigated in preliminary tests involving inhibition of growth by a number of auxins (principally 2,4-dichlorophenoxyacetic acid, 2,4-D). In none of these preliminary tests could any antagonism be detected. Then in 1949 Bonner (8) published his paper claiming this compound as an auxin antagonist. A critical survey of his data, however, showed (see next section) that other conclusions could quite logically be drawn from them and demonstrated the clear need for further study of DCA-auxin interactions in growth. The early preliminary experiments on DCA have therefore now been repeated and further data acquired from more elaborate experiments with excised pea root sections.

The Original Experiments with *Avena* Coleoptile Sections, (Bonner, 1949)

The original experiment of Bonner was a factorial experiment in which *Avena* coleoptile sections were grown in 2 per cent sucrose solutions at 25° C in different concentrations of β -indolylacetic acid (IAA) and DCA, both alone and in all possible combinations. The concentrations used were IAA at 0, 0.01, .1, 1 and 10 mg/l and DCA at 0, 2.5, 5 and 10 mg/l. Total extension was measured after 24 hours. From the results (Table 11 of his paper) he claims that analysis (by the methods of Lineweaver and Burke, 19, for competitive inhibition in enzyme systems *in vitro*) shows that »while DCA may act as a competitor of IAA at low IAA concentrations (0.01—1 mg/l) this is not true at high concentrations (1—10 mg/l)«. It is however dangerous to apply to such a variable system as a coleoptile section and to such a complex process as growth the exact methods applicable to the analysis of simple chemical systems. In this present problem a statistical treatment by the methods of the analysis of variance is preferable. If, as would seem likely from superficial scrutiny of the results, the inhibition of growth due to DCA was exerted quite independently of the stimulation due to IAA, then the interaction variance of the two treatments should not exceed significantly the residual variance due to experimental errors. Such an analysis

Table 1. *DCA and IAA interactions in Avena coleoptile growth (log₁₀ conversion).*
(Data of Bonner, 8).

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square
IAA treatments	2.8854	4	.721
DCA »0749	3	.025
Interaction (IAA \times DCA)0316	12	.00263

of variance has been done, after converting the observed measurements to logarithms (\log_{10}). This was deemed advisable since the responses are obviously logarithmic and by such a conversion the variance distribution is kept as normal as possible. The results of this analysis are seen in Table 1.

Unfortunately, owing to the absence of any replication in this experiment, there is no independent source of residual variance within the experiment itself against which to test this interaction. However, in other experiments quoted in the same paper, such replication has been carried out and yields an appropriate estimate. From his Table 1, ten comparable samples of sections grown on 1 mg/l IAA and 10 mg/l sucrose gave a variance estimate (after \log_{10} conversion) of .00031. From his Table 2, seven samples taken on different occasions in different experiments give a variance estimate of .002, illustrating the very much greater »between-experiment» variance recently confirmed by Bentley (7). Using the first of these estimates, which is presumably a true estimate of »within-experiment» variance, we find a ratio of the DCA \times IAA interaction variance to this estimated residual variance of 8.5 which gives a P just above 0.1 per cent. There is thus no doubt that DCA inhibition does depend on the IAA concentration. The point that now has to be discovered is how this interaction variance is distributed between treatments. This can best be found by calculating expected values of growth for each treatment, assuming *no* interaction, and then determining the deviations of observation from these calculated values.¹

Such values have been calculated and are plotted in Figure 1 together with the observed values. A further check on the significance of these deviations is possible by calculating $(X_{\text{obs}} - X_{\text{calc}})^2$ where X_{obs} represents the observed growth and X_{calc} , the calculated value, since this is a measure of the

¹ Expected growth for any treatment can be calculated from the formula:

$$\text{Expected growth} = \text{antilog. } (\bar{X}_I + \bar{X}_D - \bar{X})$$

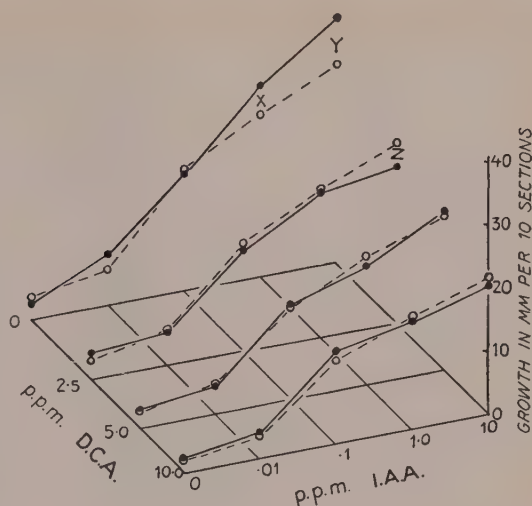
where X_I = the mean \log_{10} growth in the relevant IAA concentration both with and without DCA).

\bar{X}_D = mean \log_{10} growth in the relevant DCA concentration (both with and without IAA).

\bar{X} = grand mean \log_{10} growth (all observations).

Figure 1. Three-dimensional diagram showing the growth effects and interactions of β -indolylacetic acid (IAA) and 2,4-dichloranisole (DCA) on *Avena* coleoptile sections (from the data of Bonner, 1949).

Closed circles: Observed growth rates.
Open circles: Calculated growth rates assuming no interaction.



interaction sum of squares. This calculation yields a value of 85.02, which, with 12 degrees of freedom, gives an interaction variance estimate of 7.085. The residual variance of means calculated from his Table 1 is 0.732 giving a variance ratio of 9.7 which is again highly significant. Inspection of the figure will show however that most of this variance is due to the three deviations marked X, Y and Z; indeed calculations show that they account for nearly 88 per cent of it, the remaining deviations coming well within the experimental error. An interaction of this sort could be completely explained if the inhibitions due to DCA were greater in the higher concentrations of IAA than would have been expected if the DCA and IAA effects were completely independent. This is the reverse of what Bonner himself claimed from his results.

Methods

The objection to the use of such material as *Avena* coleoptile sections for demonstrating anti-auxin action is that one is measuring the blocking of a process for which added auxin is very largely a necessity and, unless care is exercised in interpreting results, the inhibition of this growth may be taken without further evidence as synonymous with the blocking of the promoting action of auxin. For such a blocking to be proved it has to be shown that the growth inhibition, relative to that in the absence of auxin or in low concentrations of it, is progressively relieved as the auxin concentration is increased, demonstrating thereby a definite competition for growth centres. Such a demonstration has recently been made by Leopold and Klein (17)

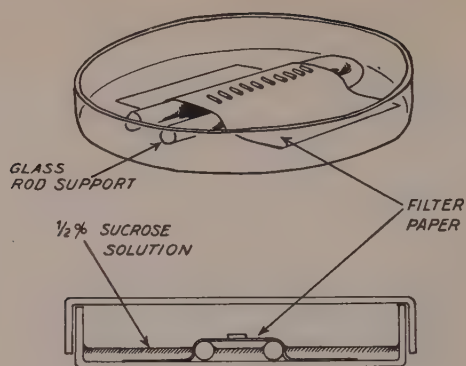


Figure 2. Method of growing excised segments from the extension zone of pea roots on 0.5 % sucrose solutions under conditions of maximum aeration. (For further explanation see text).

in the action of maleic hydrazide in the Pea test and by van Overbeek et al. (22) for trans-cinnamic acid. A much more satisfactory method of demonstrating »anti-auxin» action is its relief of the auxin inhibition of root growth. If a molecule, which by itself at certain concentrations has no action on the growth of roots, can remove, at least partially, the growth inhibition by auxins, then there can be no doubt that a direct antagonism exists between them. The site and nature of this antagonism is, however, another matter. Such antagonisms have recently been firmly established for the compound 2-naphthyl-methyl-sulphide propionic acid (NMSP) and (—) α (2-naphthoxy)-propionic acid by Åberg (1 and 2). In the following experiments a similar technique with roots has been employed to investigate the possible anti-auxin action of DCA.

Two types of experiment have been carried out. The first type, being an elaboration of the original preliminary experiments, have been based on the overall growth in length of seedling radicles of cress (*Lepidium sativum*) growing in dilute culture medium for four days after germination. The technique has been fully described in a previous publication (Audus, 5) and will not be dealt with in detail here. The growth measurements recorded represent the average length of radicles of samples of 20 seedlings measured after 5 days, and therefore correspond to the overall growth of normal attached roots.

The second type of experiment has used a modification of the technique of Brown (Brown and Sutcliffe, 11) in which 2 mm. sections excised from the extending zone of primary roots of pea (*Pisum sativum*) have been grown on 0.5 per cent sucrose solution. The extension zone of pea radicles is situated from .2 to .4 cm. behind the tip and excision is carried out from 3-day old pea seedlings by means of a special guillotine. Ten such sections constitute a sample and immediately after excision they are surface dried with filter paper and then weighed rapidly to the nearest 0.1 mg. on a suitable micro-torsion balance. They are then placed in petri-dishes and supported on filter paper bridges over the sucrose solution, to which the auxins, anti-auxins or their mixtures are added. The ends of the filter paper bridge dip into the solution and serve as a wick for its supply to the sections. The arrangement used is shown in Figure 2. In this way maximum aeration of the sections is ensured, since such sec-

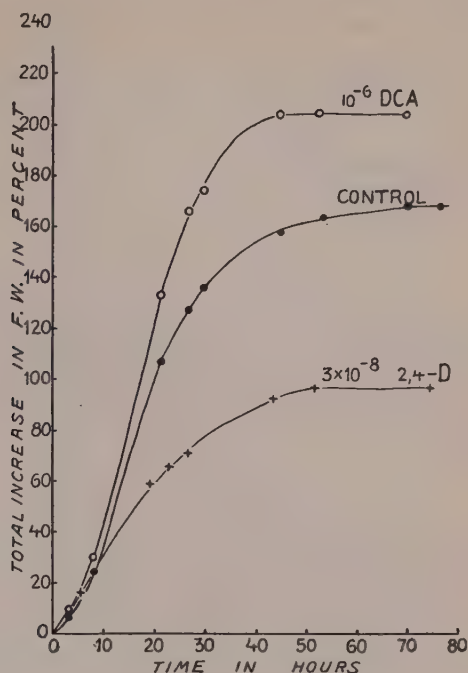


Figure 3. Typical growth curves for excised pea root sections using the weighing technique.

tions are extremely sensitive to this factor and their growth can be considerably inhibited by flooding them with liquid. They are then covered with a lid, placed in darkness in a 25° C incubator at a humidity of about 95 per cent and growth followed for 48—60 hours. At intervals (2—3 hours during the day), sections are removed at the end of a scalpel, dried rapidly on filter paper, weighed to the nearest 0.1 mg. and then replaced. This whole operation can be performed in just over 1/2 minute causing a minimum disturbance in growth conditions. This gives an accurate measure of extension growth since there is a negligible increase in diameter of the sections and most of the increase in fresh weight is thus due to extension along the main axis. Extremely smooth growth curves are thereby obtained as can be seen in Figure 3, where a selection of typical curves is reproduced.

Results. DCA Antagonisms

Cress. Total growth of radicles

1. DCA and 2,4-D inhibition

The results of an experiment in which three inhibiting concentrations of 2,4-D (i.e., .01, 0.1 and 1.0 p.p.m.) were used are reproduced graphically in

Table 2. *DCA and 2,4-D interactions in the total growth of Cress radicles, (\log_{10} conversion).*

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	P
2,4-D treatments	3.597	3	1.199	15 %
DCA »	1.269	3	.423	
Interaction092	9	.0102	
Residual165	32	.00516	

Figure 4. The closed circles represent the individual observed sample means. Obviously here too the relationship between concentration and response is logarithmic and so an analysis of variance has been carried out, as for Bonner's results, using a \log_{10} conversion. The results of such an analysis are shown in Table 2.

The interaction/residual variance ratio is 1.977 giving a probability of 15 per cent. Although this is scarcely acceptable as a significant interaction, the values of expected means, assuming no interactions, have been worked out as for Bonner's results. These means appear as open circles in Figure 4. Here it will be seen that the small interaction is very largely due to a very high growth in the concentration mixture, .01 p.p.m. 2,4-D and 1 p.p.m. DCA, suggesting antagonism. To test this further, the experiment was repeated using concentrations of 0 and .01 p.p.m. 2,4-D and concentrations of 0, 1 and 10 p.p.m. DCA and all combinations thereof. Replication was five-fold

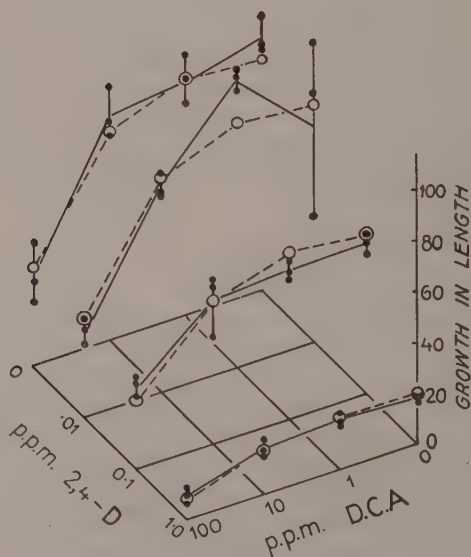


Figure 4. *Three-dimensional diagram showing the interactions in the growth inhibition of cress radicles of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dichloranisole (DCA). Closed circles: Observed mean growth in length of individual samples. Open circles: Calculated mean growth in length assuming no interaction.*

Table 3. *DCA and 2,4-D interactions in the total growth of Cress radicles.*

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	P
2,4-D treatments	333	1	338	10 0/0
DCA »	3126	2	1563	
Interaction	278	2	139	
Residual	1577	23	68.6	

Table 4. *Mean growth of five replicate samples (Cress roots)
(relative to controls in water).*

DCA conc. (p.p.m.)	2,4-D concentration (p.p.m.)	
	0	.01
0	100	84.2
1	96.4	96.0
10	72.2	71.8

with each treatment. The results of this experiment has been analysed in Table 3. No \log_{10} conversion has been applied here since all responses were very small. The interaction/residual variance ratio is 2.026 giving a probability of about 10 per cent. The nature of this small interaction is illustrated in Table 4 where the mean growths of six samples relative to controls are recorded. In the presence of both DCA concentrations, the small inhibition due to .01 p.p.m. 2,4-D is not observed, and this is explainable purely in terms of an antagonism. A probability of 10 per cent is however uncomfortably high and we must regard the DCA-2,4-D antagonism indicated here as still »not proven».

2. *DCA and 2-methyl-4-chlorophenoxyacetic acid (MCPA) inhibition*

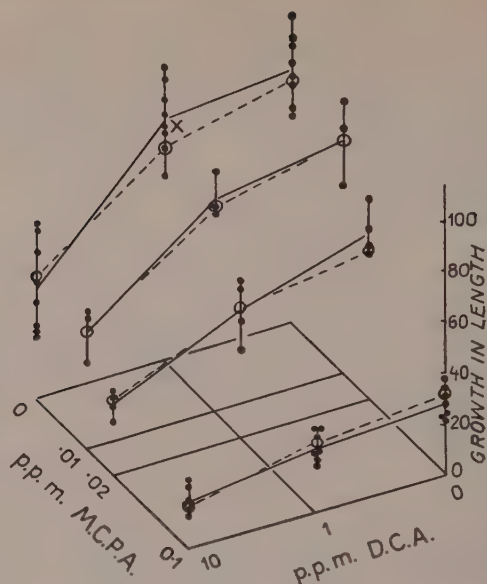
The results of experiments using three inhibiting concentrations of MCPA (.01, .02 and 0.1 p.p.m.) and two concentrations of DCA (1 and 10 p.p.m.) are shown in Figure 5. Statistical analysis of variance after a \log_{10} conversion of the data is given in Table 5. The interaction/residual variance

Table 5. *DCA and MCPA interactions in the total growth of Cress radicles,
(\log_{10} conversion).*

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	P
MCPA treatments ...	2.9284	3	.9761	10—20 0/0
DCA »9766	2	.4883	
Interaction1184	6	.0197	
Residual5547	51	.0109	

Figure 5. Three-dimensional diagram showing the interaction in the growth inhibition of cress radicles of 2-methyl, 4-chlorophenoxyacetic acid (MCPA) and 2,4-dichloranisole (DCA).

Closed circles: Observed mean growth in length of individual samples.
Open circles: Calculated mean growth in length assuming no interaction.



ratio is 1.807 giving a probability between 10 and 20 per cent. The nature of this small interaction is seen in Figure 5 where calculated means are plotted as open circles. Inspection of the figure shows that in the absence of DCA, MCPA inhibition increases more rapidly with concentration than expectation whereas in the presence of 10 p.p.m. DCA it increases less rapidly. This indicates that this small interaction is of the nature of an antagonism of MCPA inhibition by DCA but it is still well below the acceptable level of significance.

3. DCA and phenoxyacetic acid (PoXA) inhibition

Since phenoxyacetic acid (PoXA), the parent molecule of 2,4-D and MCPA, has a very much lower physiological activity than its derived compounds, it might be expected that any antagonistic action of DCA might show up

Table 6. DCA and PoXA interactions in the total growth of Cress radicles, (\log_{10} conversion).

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	P
PoXA treatments2609	2	.1304	0.1 %
DCA "0158	2	.0079	
Interaction0581	4	.0145	
Residual074	31	.00238	

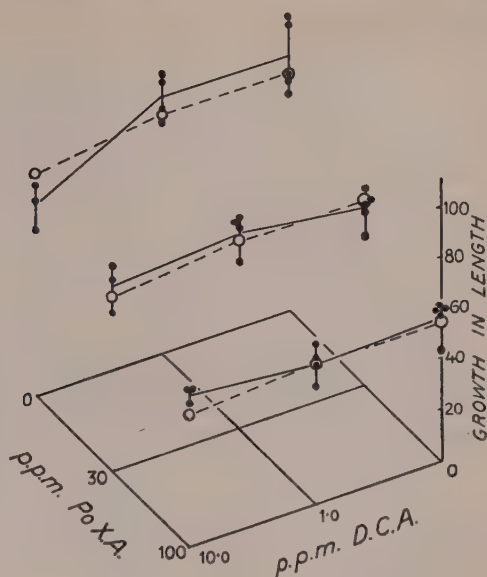


Figure 6. Three-dimensional diagram showing the interactions in the growth inhibition of cress radicles of phenoxyacetic acid (PoXA) and 2,4-dichloranisole (DCA). Closed circles: Observed mean growth in length of individual samples. Open circles: Calculated mean growth in length assuming no interaction.

most against a weakly active molecule of this nature. Consequently experiments were carried out using two slightly inhibitory concentrations of PoXA (30 p.p.m. and 100 p.p.m.) and two concentrations of DCA (1 and 10 p.p.m.). The results are shown in Figure 6, where the means calculated for no interaction are drawn as open circles. An analysis of variance of these results after \log_{10} conversion is given in Table 6. The interaction/residual variance ratio is 6.09 giving a probability of 0.1 per cent. This is a highly significant interaction and, as can be seen from Figure 6, is due to a marked flattening of the PoXA inhibition-concentration curve in the presence of 10 p.p.m. DCA. There is thus a small but undoubted antagonism of PoXA action by DCA.

Pea. Extension growth of excised root sections

1. DCA and 2,4-D inhibition of growth

In the first experiment two very markedly inhibiting concentrations of 2,4-D were chosen, (.01 p.p.m. and .03 p.p.m.) and these were used in combination with two concentrations of DCA which are just on the inhibition threshold (i.e. 1 p.p.m. and 3 p.p.m.). For one concentration combination (i.e. 3 p.p.m. DCA and .01 p.p.m. 2,4-D) this represents a ratio of »antagonist» to »hormone» of 300/1. The replication scheme is shown in Table 7.

For each of the samples growth curves were plotted and from each curve

Table 7. *DCA and 2,4-D interactions in the extension growth of Pea root sections. Plan of Experiment.**Number of samples of 10 sections.*

DCA conc. (p.p.m.)	2,4-D concentrations (p.p.m.)		
	0	.01	.03
0	6	3	3
1.0	4	2	2
3.0	4	2	2

Table 8. *DCA and 2,4-D interactions in the extension growth of Pea root sections.*

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	P
2,4-D treatments	5,931	2	2,965	
DCA »	877	2	439	
Time	32,967	4	8,242	
Interactions:-				
2,4-D \times DCA *	100	4	25	<1.0%
2,4-D \times Time	1,259	8	157	
DCA \times Time *	504	8	63	
Triple interaction * ...	952	16	59.5	
Residual	5,012	95	52.76	
Lumped Residual + Insignificant Interactions (*) }	6,568	123	53.4	

the percentage increase in fresh weight for each successive ten-hour period from 0—50 hours was read from the smoothed curves (as drawn in Figure 3). After this period growth has usually ceased.

An analysis of variance was then performed on this growth rate data. A complete analysis is shown in Table 8.

It will be seen that the only interaction that reaches significance level is 2,4-D effect with time, and this means that the 2,4-D inhibition varies with time in a manner independent of DCA concentration. The DCA effect is itself independent of time in this experiment and is an inhibition in both concentrations. The salient points of the growth relationships are brought out in Figure 7. The first graph (A) shows time changes in the growth rate in the three 2,4-D concentrations. It will be seen that in both .01 and .03 p.p.m. 2,4-D the maximum inhibition of growth rate takes place between 10 and 30 hours. Prior to this, inhibition is not so marked, since presumably the 2,4-D has not reached the maximum concentration at the site of its action in the cell (See Audus, 6). Later there is a recovery from this inhibition, sections even growing slightly faster than controls in .01 p.p.m. These dif-

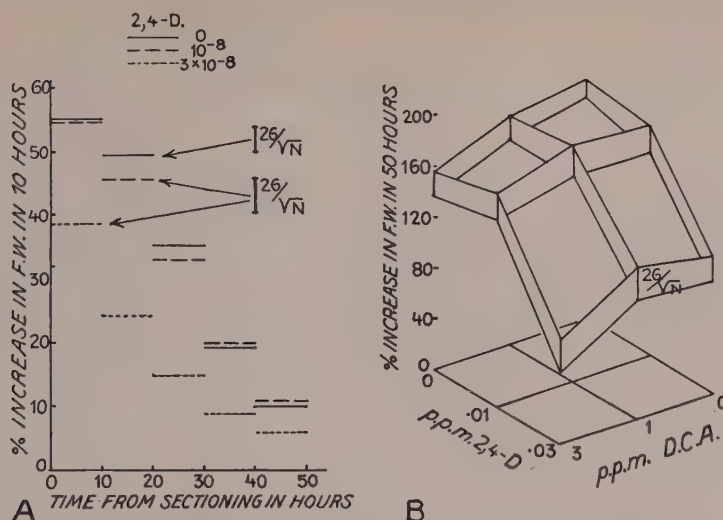


Figure 7. Growth effects and interactions of 2,4-dichlorophenoxyacetic acid (2,4-D) (High concentrations) and 2,4-dichloranisole (DCA) in excised root sections of pea.

A. Analysis of the 2,4-D \times Time interaction.

B. Analysis of the 2,4-D and DCA effects.

ferences are significant to the 1 per cent level. In graph (B) the lack of interaction between 2,4-D and DCA in the total growth after 50 hours is illustrated. The parallel lines are arranged at distances apart of twice the standard error of the means. The two substances are behaving as if they were exerting purely additive inhibitory effect.

In the second experiment on 2,4-D and DCA much lower inhibiting

Table 9. DCA and 2,4-D interactions in the extension growth of Pea root sections.

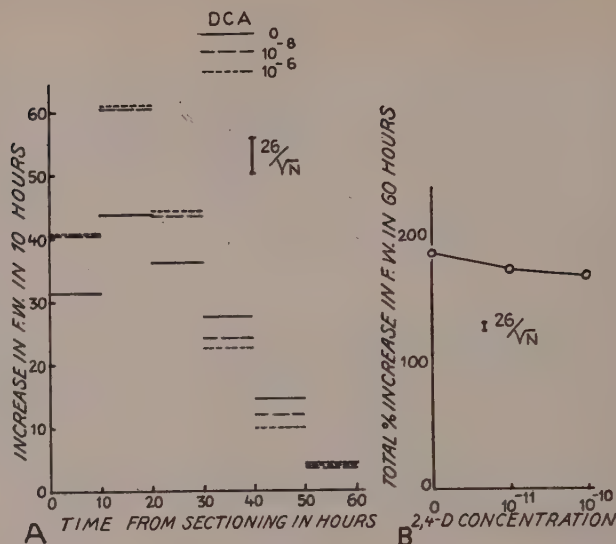
Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	P
2,4-D treatments	271	2	135	15 0/0
DCA "	684	2	342	
Time	49,503	5	9,900	
Interactions:-				
2,4-D \times DCA *	82	4	21	0.1 0/0
2,4-D \times Time	896	10	89.6	
DCA \times Time *	2,148	10	214.8	
Triple interaction * ...	612	20	30.6	
Residual	8,386	108	77.6	
Lumped Residual + insignificant Interactions (*) }	9,976	142	70.25	

Figure 8. *Growth effects and interactions of 2,4-dichlorophenoxyacetic acid (2,4-D) (low concentrations) and 2,4-dichloroanisole (DCA) in excised root sections of pea.*

A. Analysis of the DCA \times Time interaction.

B. The 2,4-D effect.

(Increase in F.W. in A given in %)



concentrations of 2,4-D were selected in the hope that the smaller inhibitions so induced might be much more easily antagonised by two concentrations of DCA. The concentrations used were 10^{-5} and 10^{-4} p.p.m. of 2,4-D and .01 and 1 p.p.m. of DCA. Samples at all concentrations and combinations of concentrations and controls in 0.5 per cent sucrose alone were replicated three times. Precisely the same procedure was adopted as for the preceding experiment for the measurement of growth rates, which were determined over 0–60 hours. The analysis of variance is given in Table 9. In this experiment the only significant interaction is DCA and time, while the 2,4-D effect at these low concentrations is on the fringe of significance. The nature of these relationships is shown in Figure 8. The graph (A) shows the nature of the DCA effect and its change with time and concentration. In both concentrations there is a marked and significant stimulation of growth rate in the first three 10-hour periods but this stimulation is followed by a depressed growth rate. Both the effect and its change with time are highly significant. These results are in strong contrast to those of the previous experiment where 1 p.p.m. DCA produced a significant inhibition of growth at all stages. This is a further illustration of a point which is a consistent feature of so much work of this kind, namely that variation between batches of living material taken and studied on different days is very high compared with the variation within batches in one experiment. It also stresses the caution that should be exercised in drawing conclusions from experiments in which this wider response has for some reason not

been studied. Figure 8 B shows the very slight 2,4-D inhibiting effect at these low concentrations. Further replication of these experiments might be expected to establish these as significant.

2. DCA and the action of IAA

In investigating the antagonism of IAA by DCA in root section growth two concentrations of IAA have been used, i.e. 10^{-5} p.p.m. and .01 p.p.m. The former concentration is that which is optimal for growth stimulation. It was felt that if such a low concentration was used with a DCA concentration just on the threshold of inhibition, (i.e. 1 p.p.m.) then the very high ratio of »antagonist» to auxin of 10^5 should demonstrate an action if one were present. The second value of .01 p.p.m. IAA was chosen since this represents a concentration just over the threshold of inhibition. This might represent also a particularly sensitive concentration region for root response where any antagonistic action by DCA might be expected to push the response over into the stimulating region of lower effective auxin concentrations. A very-wide range of DCA concentrations was chosen to ensure coverage of the proper threshold in view of the wide variation of response to this compound shown in the previously described experiment. The layout of the experiment is shown in Table 10.

As in the case of the DCA—2,4-D interaction experiments, growth curves were plotted for the whole of the 50-hour growth period and from the smoothed values the growth rates over each successive 10-hour period were calculated. An analysis of variance was then carried out and appears in Table 11. Inspection of this analysis shows that the triple interaction/residual variance ratio is 2.136 which is highly significant. This means that the interaction with which we are concerned between IAA and DCA effects is closely

Table 10. *DCA and IAA interactions in the extension growth of Pea root sections. Plan of experiment.*
Numbers of samples per treatment.

Conc. of DCA (p.p.m.)	Conc. of IAA (p.p.m.)		
	0	10^{-5}	.01
0	6	2	4
10^{-4}	3	1	2
10^{-3}	3	1	2
.01	3	1	2
.1	3	1	2
1.0	3	1	2
10.0	3	1	2

Table 11. *DCA and IAA interactions in the extension growth of Pea root sections.*

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	P
IAA treatments	1,440	2	720	about 0.1 0/o
DCA »	8,768	6	1,461	
Time	138,400	4	34,600	
Interactions:-				
DCA \times IAA	541	12	45	
IAA \times Time	2,683	8	335	
DCA \times Time	2,827	24	117.7	
Triple interaction	7,939	48	165.3	
Residual	10,455	135	77.4	

Table 12. *DCA and IAA interactions in the extension growth of Pea stem sections.*

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	P
<i>Time period 0—10 hours</i>				
IAA treatments	4,883	2	2,442	< 0.1 0/0
DCA treatments	379	6	63	10—20 0/0
Interaction	2,461	12	205	
Residual	3,394	27	125.7	
<i>Time period 10—20 hours</i>				
IAA treatments	655	2	327.5	< 10 0/0
DCA treatments	8,434	6	1,406	< 0.1 0/0
Interaction	1,480	12	123	
Residual	3,626	27	134.3	
<i>Time period 20—30 hours</i>				
IAA treatments	630	2	315	1—5 0/0
DCA treatments	4,166	6	694	< 0.1 0/0
Interaction	1,307	12	109	20 0/0
Residual	2,028	27	75.1	
<i>Time period 30—40 hours</i>				
IAA treatments	639	2	319.7	1 0/0
DCA treatments	917	6	153	< 5 0/0
Interaction	470	12	39	
Residual	1,832	27	67.8	
<i>Time period 40—50 hours</i>				
IAA treatments	324	2	162	< 0.1 0/0
DCA treatments	224	6	37	1—5 0/0
Interaction	129	12	11	
Residual	394	27	14.6	

dependent on the stage of the growth phase considered and necessitates a breakdown analysis with time. This has been done and the results appear in Table 12. The main effects and interactions that come out of this analysis are illustrated in Figure 9, where percentage growth over each 10-hour

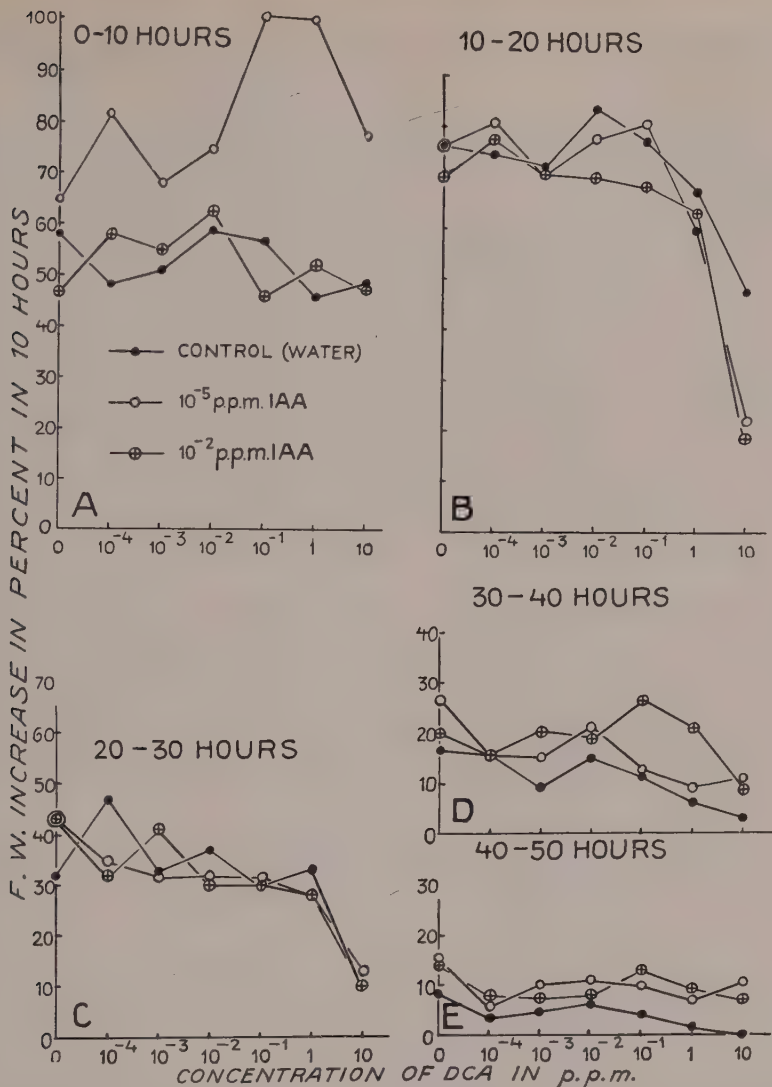


Figure 9. Growth effects and interactions of β -indolylacetic acid (IAA) and 2,4-dichloroaniline (DCA) in excised root sections of pea.

period is plotted against the DCA concentration for each IAA concentration. The significant effects are as follows:

In the first place the lower concentration of IAA brings about a large (about 60 per cent) increase in the growth rate in the first ten hours while over the same period the higher concentration has no effect. In the next two 10-hour periods this stimulation disappears, probably as a partial result

of IAA breakdown in the system, but a slight inhibition, which is just at the acceptable level of significance, appears in the higher concentrations. Later on, in the latter phases of growth, the growth rate remains at a significantly higher level in both concentrations. The reason for this prolongation of growth by the higher concentration can be explained in terms of IAA destruction, which is known to go on very actively in dilute solutions, particularly when in contact with damaged tissues (Mes, 20, and unpublished results by the senior author). Such destruction might proceed until stimulatory concentrations of IAA (10^{-5} p.p.m.) are reached in the latter stages of growth. It is difficult, however, to explain the phenomenon in the low concentrations, where it is highly probable that IAA has long since disappeared from the medium. One possible explanation is that the onset of maturation processes, culminating in the cessation of growth, have been delayed by the earlier effects of added auxin. Such maturation effects have been suspected from previous work, although as a result of applications of much higher concentrations (Audus, 4, Michel, 21).

The inhibiting effect of high DCA concentrations (1 and 10 p.p.m.) is not visible until after the first 10-hour period and this is probably a result of slowness of penetration to the growth centres (cf. page 440 and Audus, 6). Then onwards, the effect can be observed during the whole of the subsequent growth, the degree of inhibition remaining remarkably constant at about 70 per cent (for 10 p.p.m.) during the 10–30-hour period. The graph suggests that the inhibition in the last two periods may be removed by IAA but the statistical analysis shows that this interaction is not significant. Note that at no time and in neither DCA concentration is any stimulation of the growth rate visible.

The only DCA–IAA interactions that approach significance are for the 0–10 hour and 20–40 hour periods. For the former, graph 9 A shows that it is due entirely to particularly high growth rates in 10^{-5} p.p.m. IAA and 1 and 0.1 p.p.m. DCA. This is synergistic action and not antagonism. The interaction at 20–30 hours is due to the opposing tendencies of the control and .01 p.p.m. IAA curves at the 0, 10^{-4} and 10^{-3} p.p.m. DCA concentration level. This has no obvious physiological significance and can therefore be disregarded. Obviously therefore at these threshold concentrations of IAA a very wide range of DCA concentrations exert no antagonistic effects on auxin action at any stage during the whole extension period.

Results. Antagonism by 2,4-dichlorophenol

In view of the close structural similarity of DCA and 2,4-dichlorophenol this latter compound has been investigated in growth experiments with cress and the inhibition of its root growth by 2,4-D. The phenol differs from DCA in that its inhibition suddenly increases as the concentration is increased between 10 and 100 p.p.m. At the former concentration inhibition is scarcely detectable while at the latter it is complete. Only very weak phenol solutions have therefore been investigated at concentrations around the inhibition threshold. They have been studied with 2,4-D itself in concentrations just above the inhibition threshold concentration. One major experiment was carried out with 1 and 10 p.p.m. 2,4-dichlorophenol and .01 p.p.m. 2,4-D, each treatment being replicated twelve times.

The results of this experiment are shown in Tables 13 and 14. In Table 13 are recorded the means of the growth of the 12 replicates from each treatment and it will be seen that, as for DCA in the same concentration range, the inhibition by .01 p.p.m. 2,4-D is completely removed by the phenol. This interaction is not so highly significant as for DCA and 2,4-D, although by comparison with Table 4 it will be seen that the effects are of the same order. An analysis of variance of the full data is given in Table 14. The interaction/residual variance ratio is 2.69 corresponding to a probability of about 8 per cent. This high value is undoubtedly due to the very variable

Table 13. 2,4-dichlorophenol and 2,4-D interactions in the total growth of Cress radicles.

Mean growth relative to control ($\pm\sqrt{N}$).

Phenol conc. (p.p.m.)	2,4-D conc. (p.p.m.)	
	0	.01
0	100 \pm 2.36	86.5 \pm 3.72
1.0	99.8 \pm 4.90	105 \pm 4.32
10.0	94.4 \pm 4.24	92.6 \pm 4.34

Table 14. 2,4-dichlorophenol and 2,4-D interactions in the total growth of Cress radicles.

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	P
2,4-D treatments	277	1	277	8 0/0
Phenol treatments	1,308	2	654	
Interaction	1,057	2	529	
Residual	12,976	66	196.6	

plant material which happened to be used in this experiment. The residual variance for the corresponding 2,4-D—DCA interaction experiment was 68.6, i.e. just over a third that of the present experiment. The use of more uniform material of this kind in this experiment might be expected to give more highly significant interaction than for the DCA experiments. It seems highly likely therefore that DCA is no more effective in antagonising 2,4-D inhibitions than is 2,4-dichlorophenol.

Results: Interactions between auxins

Experiments have also been carried out on cress seedlings on the interactions between the auxins themselves. The results of one such experiment, i.e. between 2,4-D and phenoxyacetic acid are recorded below. The plan of the experiment is shown in Table 15. The results appear in Figure 10 and the analysis of variance in Table 16. It will be seen that the interaction variance here is highly significant and Figure 10 shows that it is largely due to antagonism of 2,4-D inhibition by 0.1 to 10 p.p.m. of phenoxyacetic acid (PoXA). The theoretical curves of no interaction are all humped in this PoXA concentration range, due to a similar humping of the majority of the observed points in the solutions containing 2,4-D. No such humping is seen in the control sets containing no 2,4-D. All the observed values for growth in water controls lie well above the calculated mean and at the same level as those in .1, 1.0 and 10 p.p.m. PoXA alone. The one major discrepancy from this general picture is for the results in the .01 p.p.m. 2,4-D and 1 p.p.m. PoXA mixture where the observed points fall well below the calculated. Only one of these three points falls outside the accepted limits of scatter about the calculated mean and it can be shown that this point contributes only about

Table 15. *PoXA and 2,4-D interactions in the total growth of Cress radicles. Plan of experiment.*
Number of replications.

PoXA concs. (p.p.m.)	2,4-D concentration (p.p.m.)			
	0	.01	0.1	1.0
0	6	6	6	6
0.1	3	3	3	3
1.0	3	3	3	3
10	5	5	5	5
100	3	3	3	3
1,000	3	3	3	3

Table 16. *PoXA* and 2,4-D interactions in the total growth of *Cress* radicles, (\log_{10} conversion).

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	P
2,4-D treatments	586.3	3	195.4	< 0.1 %
PoXA »	1,759.0	5	351.8	
Interaction	181.6	15	12.11	
Residual	107.4	68	1.579	

2.5 per cent to the total interaction variance as determined from the total sum of squares of differences between observed points and calculated means. This humping means therefore that, in these concentrations of PoXA, the inhibitions due to 2,4-D are much smaller than those in the absence of PoXA, i.e. a definite antagonism. Similar experiments with 2,4-D and IAA, and 2,4-D and MCPA showed no similar interaction between these hormones of high activity, and their inhibitions are therefore, within the limits of experimental error, additive. In this experiment then we have a definite proof of the antagonism of the action of a strong auxin by a weak one.

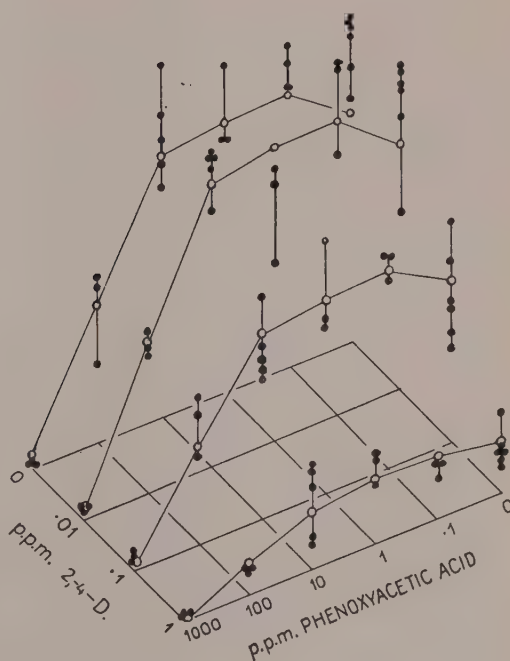


Figure 10. Three-dimensional diagram showing the interactions in the growth of cress radicles of phenoxyacetic acid (PoXA) and 2,4-dichlorophenoxyacetic acid (2,4-D).

Closed circles: Observed mean growth in length of individual samples.

Open circles: Calculated mean growth in length assuming no interaction.

On the ordinate: Growth in length.

Discussion

The final outcome of the experiments that have been described is to make it doubtful whether 2,4-dichloroanisole can be regarded as an effective »anti-auxin«. Only in one instance has an interaction with a growth hormone reached a desirable level of significance ($P < 5$ per cent). This was with phenoxyacetic acid, the very weakly inhibiting action of which in cress root growth was very slightly relieved by the DCA. There were indications that similar slight antagonisms were present against 2,4-D and MCPA inhibition in the same material and that these might reach an acceptable level of significance if more uniform plant material could be found to work upon. On the other hand a precisely similar indication was obtained with 2,4-dichlorophenol interaction with 2,4-D. These latter results resemble the much more positive ones of Åberg (1) in which the antagonistic action of α -naphthol was demonstrated with several auxins. The degree of all these antagonisms was moreover very small and they occurred mostly at those critical concentrations of auxins just above the threshold of inhibition.

On the other hand no trace of an antagonism between DCA and either β -indolylacetic acid or 2,4-D could be evoked using sections excised from the extending zone of pea radicles, grown in a 0.5 per cent sucrose. Here the only trace of an interaction was the suggestion of a synergistic action of DCA at 0.1 and 1 p.p.m., augmenting the IAA stimulation of the early phases of growth at 10^{-5} p.p.m. It did not however reach an acceptable level of significance. A somewhat different synergism was indicated by a re-analysis of Bonner's original data for *Avena* coleoptile sections, where a significant interaction is demonstrable, and this takes the form mainly of a heightened sensitivity of the coleoptile sections to DCA inhibition in the highest auxin concentrations.

The fact that in some material (some samples of pea) DCA itself can behave precisely as does IAA, by stimulating in low concentrations (.01 and 1.0 p.p.m.) the early stages of extension growth of sections and inhibiting growth above those concentrations, indicates that we may have to revise drastically our concepts of the rôle of auxins and particularly IAA in root growth. It is of course possible to explain the above DCA stimulation of root growth in terms of antagonism of the natural auxin (often assumed to be IAA), which has for a long time been regarded as being present in supra-optimal inhibiting concentrations. The relief of this inhibition by DCA could account for the observed stimulation of growth. This is the explanation put forward by both Burström (12) and by Åberg (1) to explain similar stimulations of root growth by compounds such as NMSP and α -naphthoxy-propionic acids. But these results merely serve to throw up in stronger relief the

enigmatical position of IAA which has precisely the same type of action on elongation growth of roots as these antagonists. If IAA is the natural hormone in roots and if it is present in supra-optimal concentrations, then very elaborate and unconvincing theorising is necessary to explain the augmented growth when further small amounts are supplied in the external medium (Burström, 12). Might it not be that IAA is not the natural root hormone holding growth below its possible maximum?

This suggestion might seem highly heretical to many since our ideas on the hormone physiology of elongation growth in roots have always been dominated by facts borrowed from the study of coleoptiles and stems and indirect evidence uncritically accepted from the study of roots themselves. The writers are not aware that any indisputable evidence exists for the occurrence of IAA in roots or that auxins, as we know them in coleoptiles and stems, play any part in the normal regulation of stretching growth in roots. The observations that concentrations of IAA of the order of one part in 10^{10} or 10^{11} , when applied *externally*, accelerate elongation growth, have been interpreted quite unjustifiably as an «auxin action». Why can we not include such substances as NMSP in the category of auxins since they bring about a similar stimulation of growth in low concentrations? The reason so far put forward is based on the demonstration of their antagonism of the inhibiting action of *externally applied auxins*, where antagonism of mere access to the growth system is not excluded as a possible explanation of the effects observed. The argument is also based on the assumption, largely disproved by certain independent investigations (Gorter, 15; Younis, 25), that «auxins» are present in supra-optimal concentrations in roots. The fallacious nature of the argument is further stressed by the fact that cell poisons such as sodium arsenate (Audus, 6) and iodoacetic acid (Commoner and Thimann, 14) can stimulate extension growth in pea stems etc. in sufficiently low concentrations. Are these latter molecules then to be regarded as auxins?

To resolve these difficulties of interpretation it is better that we dispense with all preconceived notions derived from the study of stems and coleoptiles and consider the facts as they appear in relation to extension growth in roots only. Here we can distinguish two types of active compound.

(a) Those that augment growth in low concentrations and inhibit it progressively in high. Examples are IAA, NMSP and its homologues (Åberg, 1), isobutyric acid homologues of the auxins (Burström, 13), etc.

(b) Those that only inhibit growth in all concentrations. Examples are most of the synthetic auxins that have so far been investigated in a wide range of concentrations, i.e. 2,4-D, naphthoxyacetic acid and phenoxyacetic acid.

With this classification of active substances we can now resolve the enigma posed by the previous classification, which was why the application of both growth stimulant (IAA) and its antagonist (NMSP etc.) should, in one and the same system, produce independently the same growth response. To do this we merely postulate that the natural growth substance present in roots is not IAA but a simple inhibitor normally maintaining extension growth below its maximum possible level. IAA, NMSP, etc., i.e. those compounds in category (a) above, are all antagonists of this inhibitor and in very low concentrations, where they themselves have no other physiological action, they release the root from this inhibition and give augmented elongation growth. Naturally the effectiveness of the molecule will vary with its structure and optimum concentrations for antagonism will naturally vary in accordance with this. The second category of compound (2,4-D, etc.) do not antagonise the natural inhibitor so that no stimulation of growth can be evoked in low concentrations.

If IAA were a direct growth stimulant in low concentrations, then we should expect antagonists to reduce its stimulating action in those concentrations. In actual fact in pea roots, DCA, which can itself stimulate growth in low concentrations, has no such neutralising action but instead there is a suggestion that it may in suitable concentrations, augment this IAA stimulation. This behaviour is more easily explained in terms of their identical action in the same system, which, on the above hypothesis, would be the antagonism of the unknown natural inhibitor.

In high concentrations of both types of compound, root growth inhibition sets in and the actual site of this inhibiting action in the growth system may be quite different from that at which the naturally occurring inhibitor works. It is this high-concentration inhibition by the *externally applied* auxins that runs parallel with auxin action in the extension growth of coleoptiles and shoots, highly active auxins being very effective inhibitors (e.g. IAA and 2,4-D) weak auxins having small inhibitory powers (e.g. PoXA). By endowing these inhibiting molecules with two distinct and differing properties, *affinity* for the growth system in which inhibition is exerted and *activity*, which is a measure of the effectiveness of the molecule when present at the inhibited centre, Åberg (3) has been able to explain the antagonism of a strong auxin inhibitor (e.g. 2,4-D) by a weak one (e.g. phenylacetic acid) as well as similar antagonisms by the special category of so-called »auxin-antagonists» (e.g. NMSP). Similar reasoning would explain the observations in this paper of the antagonism of 2,4-D inhibition by PoXA. The two types of antagonism, i.e. of the naturally occurring inhibitor and of the auxin applied externally might, as has already been

intimated, most probably be associated with different aspects of the chemical configuration of the antagonising molecule.

It would be premature to elaborate this theory further to account for a possible natural rôle of IAA in the extension growth of roots. This should properly await a convincing demonstration that this molecule does in fact normally occur there.

In the physiology of root growth a study of antagonisms between growth-active substances will undoubtedly go a long way to solving some of the problems that now beset us in this sphere of hormone investigations, but substances showing much more active antagonisms will have to be sought. Research along these lines is being actively pursued in this laboratory. The above experiments and those that have so far appeared in the literature show, however, that little if anything can be expected of 2,4-dichloranisole as an antagonist of the auxins.

The authors wish to express their appreciative thanks to Mr. Patrick Slater of the Social Studies Department of Bedford College for his valuable advice and criticism during the statistical analyses of the data presented in this paper.

Summary

1. Owing to the unsatisfactory nature of the experimental data put forward to support the claims that 2,4-dichloranisole (DCA) is an auxin antagonist, extensive experiments have been carried out with this molecule in an attempt to antagonise the auxin inhibition of root growth.

2. Adequately replicated experiments designed to yield results capable of precise statistical analysis were performed on two types of material, attached radicles of cress (*Lepidium sativum*) seedlings and excised sections from the extension zone of pea (*Pisum sativum*) radicles.

3. A wide range of DCA concentrations was used in combination with a similar wide range of concentrations of both strong (IAA, 2,4-D, MCPA) and weak (PoXA) auxins.

4. In the inhibition of both cress and pea root growth by the strong auxins DCA showed no interaction that reached an acceptable level of significance.

5. Only in the inhibition of cress root growth by the weak auxin PoXA did DCA show a very small but statistically significant antagonism.

6. There are indications that 2,4-dichlorophenol may be a very weak antagonist of 2,4-D action in cress root growth inhibition.

7. The weak auxin PoXA can act as a feeble antagonist of 2,4-D action in cress root growth inhibition.

8. Views are expressed on the possible nature of auxin action in the control of root growth in the light of these and other observations on auxin-anti-auxin interactions.

References

1. Åberg, B.: On auxin antagonists and synergists in root growth. — *Physiol. Plant.* 3: 447. 1950.
2. — On the growth substance activity of enantiomorphic α -(naphthoxy)propionic acids. — *Arkiv för Kemi.* 3: 549. 1951.
3. — On the effects of weak auxins and antiauxins upon root growth. — *Physiol. Plant.* 5: 305. 1952.
4. Audus, L. J.: Studies on the phytostatic action of 2,4-dichlorophenoxyacetic acid and coumarin. The reversibility of root-growth inhibitions. — *New Phytol.* 47: 196. 1948.
5. — The biological detoxication of hormone herbicides in soils. — *Plant and Soil.* 3: 170. 1951.
6. — The time factor in studies of growth inhibition in excised organ sections. — *J. exp. Bot.* 1952 (In the press).
7. Bentley, J. A.: An examination of a method of auxin assay using the growth of isolated sections of *Avena* coleoptile in test solutions. — *J. exp. Bot.* 1: 201. 1950.
8. Bonner, J.: Limiting factors and growth inhibitions in the growth of the *Avena* coleoptile. — *Amer. J. Bot.* 36: 323. 1949.
9. — Further experiments on flowering in *Xanthium*. — *Bot. Gaz.* 110: 625. 1949.
10. — & Thurlow, J.: Inhibition of photoperiodic induction in *Xanthium* by applied auxin. — *Bot. Gaz.* 110: 613. 1949.
11. Brown, R. & Sutcliffe, J. F.: The effects of sugar and potassium on extension growth in the root. — *J. exp. Bot.* 1: 88. 1950.
12. Burström, H.: Studies on growth and metabolism of roots. IV. Positive and negative auxin effects on cell elongation. — *Physiol. Plant.* 3: 277. 1950.
13. — Studies on growth and metabolism of roots. VI. The relative growth action of different isobutyric acid derivatives. — *Physiol. Plant.* 4: 470. 1951.
14. Commoner, B. & Thimann, K. V.: On the relation between growth and respiration in the *Avena* coleoptile. — *J. gen. Phys.* 24: 279. 1941.
15. Gorter, C. J.: Groeistofproblemen bei Wortels. — Thesis for Doctorate in Science in the University of Utrecht. 1932.
16. Galston, A. W.: The effect of 2,3,5-triiodobenzoic acid on the growth and flowering of Soya beans. — *Amer. J. Bot.* 34: 356. 1947.
17. Leopold, A. C. & Klein, W. H.: Maleic hydrazide as an antiauxin in plants. — *Science.* 114 (2949): 9. 1951.
18. — and Thimann, K. V.: The effect of auxin on flower initiation. *Amer. J. Bot.* 36: 342. 1940.
19. Lineweaver, H. & Burk, D.: The determination of enzyme dissociation constants. — *J. Amer. chem. Soc.* 500 & 658. 1934.
20. Mes, M. G.: Prog. Rep. Plant. Phys. Res. Inst. Univ. Pretoria, S. A. 1950—51.
21. Michel, B. E.: Effects of indoleacetic acid upon growth and respiration of kidney beans. — *Bot. Gaz.* 112: 418. 1951.

22. van Overbeek, J., Blondeau, R. & Horne, V.: Trans-cinnamic acid as an anti-auxin. — Amer. J. Bot. 38: 589. 1951.
23. Veldstra, H. & Havinga, E.: Untersuchungen über pflanzliche Wuchsstoffe. VII. Über Struktur und Wirkungsmechanismus der pflanzlichen Wuchs- und Remmstoffe. — Rec. trav. chim. Pays-Bas. 62: 841. 1943.
24. Wain, R. L. 1951: Personal communication.
25. Younis, A. F.: The effect of gravity on the growth of roots. — Thesis for the Ph. D. degrees, University of London. 1950.

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P. BOYSEN JENSEN

On Volume Enlargement and Work Expenditure by an Osmotic System in Plants

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In earlier works (1, 3, 4), some thermodynamic relationships among factors of osmotic systems in plants were evaluated. These depended entirely upon the initial and final states of the system. The process path was not concerned (3, 4). Where volume changes obtain, the process path is also concerned and work expenditures can be evaluated. In the present study, factors concerned with enlargement are discussed and computations are made of the expended work of enlargement for a system assumed to be typical of the plant (1).

From equation 28 (3),

$$-(\bar{f}_e - \bar{f}_i)_1 = \bar{v}_1^{\circ} (p_e - p_i), \quad (1)$$

in which $(\bar{f}_e - \bar{f}_i)_1$, in the two component solute-solvent system, is the partial molal free energy difference of the solvent water between the external (subscript e) and internal (subscript i) phases of the system; \bar{v}_1° is the partial molal volume of water in ideal solution; and $(p_e - p_i)$ is the pressure difference on the two phases necessary to establish equilibrium between phases with respect to tendency for water flux. This pressure difference is equal to, but not identical with, the net influx intensity (net diffusion pressure deficit) for water at any time t . Further, under ideal conditions but only here, $(p_e - p_i)$ at water equilibrium is equal to the hydrostatic pressure difference between the two phases and equal to, but not identical with, the concomitantly effective hydrostatic energy intensity of flux between phases. What the net internal hydrostatic pressure or concomitant energy intensity may be or become is thus determined by the specific free energy difference of

water (net influx specific free energy or net diffusion pressure deficit) due to the presence of solute (in the simplest case) in either or both phases of an osmometer. Here, process path and volume change are not concerned.

Tendency toward distension through stretching of a limiting boundary is caused by any differential increment between the internal hydrostatic pressure and the external pressure (4); each is the resultant of pressure influences of intrinsic (P') and any extrinsic (P'') origin (1, 4). These pressures are exactly counteracted, at all times, by oppositely directed pressures of an interposed boundary (4). Enlargement of the internal phase and distension of a limiting boundary are made effective at any time t , through a finite net flux of water from the external to the internal phase. The rate of net influx is equal to the product of a flux coefficient and the energy intensity gradient for water across the membrane or boundary interposed between phases (3). The extent of enlargement at equilibrium is determined by the net influx intensity and by the elasticity and the plasticity (7) of the limiting boundary.

Levitt (10) has described the relationships between internal or external pressures and wall pressure as required by Newton's third law of motion, and between internal and external pressures, the resultant of which causes tendency toward enlargement or the opposite effect. A similar treatment was made elsewhere (4). It is only recently that particular attention has been drawn to the specific relations between the pressures concerned. One discussion (9 a) suggested a difference of opinion (compare 9 b, 10, 16, 17). Deliberation indicates that this seeming difference can be resolved by more explicit definition of the terms used. In some cases the phrase wall pressure has been used in a generic or more general sense (e.g., 2, 5, 7, 9 a) while in others it has been used in a more specific way (e.g., 4, 6, 9 b, 10, 12, 16). Assuming this variable usage of the wall pressure term, no disparity of opinion would exist among investigators except where it is proposed that the turgor pressure and wall pressure »may attain any value independent of the other« (5, 7).

When matter is acted on by forces causing tendency toward change of its size or shape it is said to be under stress, and the accompanying change in the body is called the strain. Stresses are measured by forces per unit surface and strains by changes in unit dimension. In small strains of elastic bodies the stress is proportional to the strain. The elasticity is measured by the ratio of the stress to the corresponding strain. In distention of the walls of cells by stretching, an elastic modulus is concerned somewhat similar to that for a small mechanical deflection at the middle of a beam fixed at both ends and loaded at the middle, except that the breadth of the object is not delimited. The modulus of the material of the wall may be thus (8, 10, 14,

15) evaluated. While consideration may be directed toward this characteristic of the limiting boundary material, and data of this sort have been reported (14, 15), equal interest lies in the estimation of a coefficient characteristic of the cell as a whole, namely, its coefficient of enlargement.

The increased stress in the process of enlargement of an inclosed internal phase of an osmotic system is the increase of intrinsic external pressure (boundary equivalent pressure, 4), effective inwardly; and, assuming any extrinsic external pressure components to be constant, is equal to the hydrostatic pressure increase within the phase, effective outwardly on the phase boundary. The concurrent strain in the boundary is the change in unit area of surface of the boundary, coincident with the internal hydrostatic pressure increase during enlargement. The coefficient of distention of the boundary is given by α , in pressure dimensions, in

$$\alpha = \frac{\text{stress}}{\text{strain}} = \frac{\Delta P_e'}{\frac{\Delta A}{A}} \quad (2)$$

where $\Delta P_e'$ is the intrinsic external pressure change and $\frac{\Delta A}{A}$ is the concurrent change in unit area of surface of the boundary. The boundary is assumed to be isotropic and of constant uniform thickness for small changes.

For any actual osmotic process, interest lies in the change in unit volume or enlargement of the internal phase rather than that of distention of unit surface of the boundary. The relations among the pressure change, the coefficient of enlargement, and the unit volume change are defined by

$$\epsilon = \frac{\Delta P_e'}{\frac{\Delta V}{V}}, \quad (3)$$

for any finite enlargement. Here, $\frac{\Delta V}{V}$ is the change in unit volume of the internal phase or of the cell, assuming the two to be proportional; and ϵ is the coefficient of enlargement, measured in pressure dimensions. The intrinsic external pressure change can be measured by the concurrent intrinsic internal hydrostatic pressure change, thus, $\Delta P_{h_i}'$ may be substituted for $\Delta P_e'$ in equation 3, i.e.,

$$\epsilon = \frac{\Delta P_{h_i}'}{\frac{\Delta V}{V}}. \quad (4)$$

The coefficient of enlargement of equation 3 is related to the coefficient of boundary distention of equation 2, through the relationship

$$\epsilon = \alpha \times \beta, \quad (5)$$

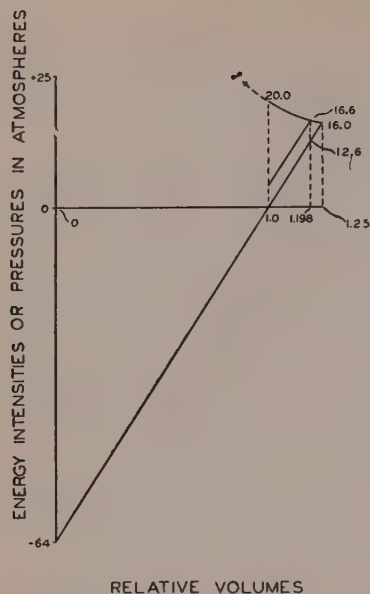


Figure 1. *Osmotic relations of systems assumed to be approximately representative of situations in plants. This figure is merely an extension of data presented earlier in more detailed form (1).*

where β is a secondary coefficient characteristic of the geometrical relations of the enlargement. Thus, for spherical or cubical enlargement, for example, β equals $2/3$.

In finding a relationship between $\frac{\Delta A}{A}$ and $\frac{\Delta V}{V}$ of a sphere, the equations used are

$$V + \Delta V = 4/3 \pi (r + \Delta r)^3, \text{ in which } r \text{ is the radius,}$$

and

$$A + \Delta A = 4 \pi (r + \Delta r)^2$$

which can be solved only approximately. The solution is simplified, however, if the infinitesimal case is considered. Thus

$$dV = 4 \pi r^2 dr$$

and

$$dA = 8 \pi r dr.$$

Then,

$$\frac{dV}{V} = \frac{4 \pi r^2 dr}{\frac{4}{3} \pi r^3} = \frac{3 dr}{r}$$

and

$$\frac{dA}{A} = \frac{8 \pi r dr}{4 \pi r^2} = \frac{2 dr}{r}.$$

Therefore,

$$2 \frac{dV}{V} = 3 \frac{dA}{A},$$

or

$$\frac{dA}{A} = 2/3 \frac{dV}{V}.$$

For the simplified integrated osmometer system of the plant (1 fig. 2), or »open» system, β is unity. Here, the enlargement is essentially in the direction of height or length, the unit volume change being directly proportional to unit change of height above the reference membrane level.

For cylindrical enlargement, the solution of β is more complex. In this case, for less complexity, it may be assumed that the enlargement is merely in the direction of length, or of diameter. In any real case, the actual relations between these must be known and simultaneously evaluated. For enlargement of more complex units or heterogeneous bodies, the mathematical solution is very difficult.

Equation 4 may be reset such that

$$\epsilon = \frac{\frac{dPh_i'}{dV}}{V} = \frac{dPh_i'}{dV} \times V = S \times V, \quad (4a)$$

in which ϵ is the coefficient of enlargement; dPh_i' is the differential pressure change, equal to $Ph_{i'2} - Ph_{i'1}$ (subscripts 2 and 1, here, indicate the differential pressure states); dV is the corresponding differential volume change, equal to $V_2 - V_1$ (subscripts 2 and 1, here, indicate the differential volume states); and V is the volume of the phase at any time t . It may be observed that $\frac{dPh_i'}{dV}$ is the slope (S) of the intrinsic internal hydrostatic pressure curve with respect to volume of the internal phase. It is evaluated, from the tangent at any point on the curve, by the ratio of the differences between the tangential coordinates of $Ph_{i'}$ and of V , viz.,

$$S = \frac{Ph_{i'2} - Ph_{i'1}}{V_2 - V_1}. \quad (6)$$

For the material of the wall, $\frac{\text{stress}}{\text{strain}}$ equals a constant where Hooke's law holds. In writing $\epsilon = \frac{dPh_i'}{dV} \times V$, the volume of the stretching material is not considered, but rather, the volume of the internal phase. For small enlargements, where V is the initial or reference volume, the coefficient of enlargement is not expected to be a constant except where β is a constant (vgl. equation 5). Constancy of β obtains for only a few geometric forms, e.g., open systems, cubes, spheres, etc. This statement for the coefficient ϵ , also applies for small volume changes at any particular volume other than the initial, during finite enlargement. However, another value of α is concerned. Rather than testing the validity of Hooke's law for differential changes of volume, consideration is directed toward evaluation of the enlargement coefficient over finite changes in volume of the phase. Here, over progressive values of

internal volume, the coefficient of enlargement is not expected to be a constant, even where Hooke's law holds, since α is a variable. The coefficient ϵ is a variable for all cases except for the situation where $\frac{dPh_i'}{dV} = \frac{k}{V}$ at progressive states of the system.

Referring to the exemplified pressure: volume relations expressed in the text of (1) and in the graphic representation thereof in figure 3 (ibidem) and figure 1 here, the relations for equations 4 and 4 a may be evaluated. According to the data presented, Ph_i' is a linear function of V so that

$$Ph_i' = (S \times V) + C, \quad (7)$$

in which S is a constant slope and C is a constant equal to the pressure intercept where $V=0$. The latter may be evaluated by extrapolation of the $Ph_i' : V$ curve. Where relative volumes are plotted, $S = -C$ always, extrapolation being unnecessary. Where the external medium is water alone, the coefficient of enlargement, at relative volume V_2 equal to 1.25, is obtained from equations 4 and 4 a after evaluation of the constants of the curve. Thus,

$$S = \frac{Ph_{i'2} - Ph_{i'1}}{V_2 - V_1} = \frac{16.0 - 0}{1.25 - 1.0} = 64, \text{ and}$$

$C = -64$. Then Ph_i' , at any value of V_2 , equals $S (V_2 - 1)$ from equation 6, i.e.,

$$Ph_i' = S (V_2 - 1) = 64 (1.25 - 1) = 16 \text{ atmospheres;}$$

or may be computed more generally, where the volumes are expressed in either relative or absolute terms, from equation 7, i.e.,

$$Ph_i' = (S \times V) + C = (64 \times 1.25) + (-64) = 16 \text{ atmospheres.}$$

ϵ may now be calculated, e.g.,

$$\epsilon = \frac{dPh_i'}{dV} \times V = S \times V = 64 V.$$

Thus, at $V_2 = 1.25$, $\epsilon = 80$ atmospheres; while at $V_1 = 1.00$, $\epsilon = 64$ atmospheres.

It was assumed, for simplicity (cf. 9), that Ph_i' is a linear function of V ; $d\epsilon$ thus also becomes a linear function of V . Experimental data with *Nitella* (14) indicate that this assumption is valid over at least a part of the range of enlargement, particularly for a range of volume change of magnitude similar to that chosen for exemplification.

Similar computations of the coefficient of enlargement can be made where the external medium is a solution, e.g., where the specific free energy of water due to solute in the external medium (frequently termed osmotic pressure of e) equals 4.0 atmospheres. Here, the slope is assumed to be the same as before (1), i.e., equal to 64. Again, where relative volumes are con-

Since $V_a = V_o \times V_r$, and $dV_a = V_o dV_r$ or $\frac{dV_r}{dV_a} = \frac{1}{V_o}$,

then

$$\frac{\varepsilon_a}{\varepsilon_r} = \frac{V_o \times V_r}{V_r} \times \frac{1}{V_o} = 1.$$

Computation of the work of enlargement concerns both the process path and the external pressure designated by P_e (4). The work of enlargement of the internal phase of an osmometer under ideal conditions is given by

$$dW = P_e \times dV \quad (8)$$

and, integrating at constant pressure (i.e., the pressure is independent of volume), by

$$W = \int_{V_1}^{V_2} P_e \times dV. \quad (8a)$$

Assuming that Ph_i differs only infinitesimally from P_e in equation 8 a, then

$$W = \int_{V_1}^{V_2} Ph_i \times dV. \quad (9)$$

This relationship has been used (7, pp. 177—178) to compute the work of enlargement where osmotic relations with time were such that the hydrostatic pressure of the internal medium remained approximately constant. For a process of enlargement where the external pressure varies concurrently with volume such that with time each successive state approaches an equilibrium state, the expended work of the system can be represented by an area subtended by a curve on a $P : V$ diagram. Here,

$$W = \int_{V_1 P_{e1}}^{V_2 P_{e2}} P_e \times dV \quad (10)$$

or, assuming that Ph_i differs only infinitesimally from P_e in equation 10, then

$$W = \int_{V_1 Ph_{i1}}^{V_2 Ph_{i2}} Ph_i \times dV \quad (11)$$

(11, pp. 53—54, 13, pp. 20—22; compare 5, 50, par. 1, and 7, pp. 177—178). The value of the integral (eq. 10 or 11) is given by the area under the $P : V$ curve. Its magnitude is determined by the particular path traversed, the characteristics of which are represented graphically in the shape of the $P : V$ curve. Work is measured in energy dimensions.

Referring to the exemplified pressure: volume relations expressed in the test of (1) and in the graphic representation thereof in figure 3 (*ibidem*) and figure 1 here, the relations for equation 11 can be evaluated. Where at each moment, as is assumed here, the external pressure and the internal hydrostatic pressure differ only infinitesimally in magnitude, values of the latter

may be used for the former. The total area under the hydrostatic pressure curve in the osmotic diagrams (loc. cit., assuming that $P_e = P_{hi}$), is a measure of the work of enlargement in liter atmospheres, where the pressure is expressed in atmospheres and the volume in liters. Other areas in an osmotic diagram bear no thermodynamic significance. The work may be evaluated by summing the coordinate areas either mathematically or graphically, but more exactly by the former. Substituting $(S \times V) + C$ from equation 7 for P_e in equation 10,

$$W = \int_{V_1}^{V_2} [(S \times V) + C] \times dV, \quad (12)$$

and integrating from relative volume $V_1 = 1.0$ to relative volume $V_2 = 1.25$,

$$W = [1/2 S \times V^2] + [C \times V] \Big|_{1.0}^{1.25} \quad (12 a)$$

$$W = \{[1/2 \times 64 \times (1.25)^2] + [(-64) \times (1.25)]\} - \{[1/2 \times 64 \times (1)^2] + [(-64) \times 1.0]\}$$

$$W = 2.0 \text{ liter atmospheres.}$$

Since the internal hydrostatic pressure curve is assumed to be linear over the volume range 1.0 to 1.25, the integral coordinate area or work is more simply given by

$$W = 1/2 (\Delta P_e) \times (\Delta V) \quad (13)$$

$$W = 1/2 (16.0) \times (0.25) = 2.0 \text{ liter atmospheres.}$$

While equations 12 and 12 a are limited to those cases where linearity of pressure change is approached, equation 10 is general and the work function can be evaluated for any case where the equation of the curve is known in terms of P_e ($\cong P_{hi}$).

Similar computations of the total work expended in enlargement of the internal phase can be made where the external medium is a solution, e.g., where the specific free energy of water due to solute in the external medium (frequently termed osmotic pressure of e) equals 4.0 atmospheres. Here,

$$W = [1/2 S \times V^2] + [C \times V] \Big|_{1.0}^{1.97}$$

or $W = 1.25$ liter atmospheres, compared with 2.0 liter atmospheres for the case of water alone in the external medium. Here also, W simply equals $1/2 (\Delta P_e) \times (\Delta V)$, i.e.,

$$W = 1/2 (12.6) \times (0.197) = 1.25 \text{ liter atmospheres.}$$

Again, values have been selected for simplicity of discussion to be representative of both enclosed and »open» systems. However, as discussed earlier

for coefficients of enlargement, more typical relations may be expressed for work expenditures with »open» systems. Osmotic relations for a typical »open» system are produced in figure 2. Here, the work expenditure in enlargement (see computations earlier for the enlargement coefficients) of an internal phase is similarly computed by means of equations 11 and 12, or 13, where $S=1.0$, $V_1=1.0$, $V_2=3.0$, and $C=-1$. Thus, W is found to equal 2.0 liter atmospheres.

It was shown earlier that the values of the enlargement coefficient were absolute for all cases including those where volumes are expressed in either relative or absolute terms. In contrast, work expenditure is actual or absolute only where the volumes are expressed in absolute terms. Where the volumes are expressed in relative terms, the work expenditure is also expressed in relative values. Thus, for calculated relative work,

$$W_r = \int P_e \times dV_r \quad (8\ b, 10\ a)$$

in which W_r is the relative work and V_r is the relative volume. In order to establish actual magnitudes for work expended in an enlargement of an internal phase, the volumes must be delimited in absolute terms as well. Thus, since $V_a = V_o \times V_r$, in which V_a is the absolute volume, V_o is the volume at $P_e=0$, and V_r is the relative volume, and since actual useful work is given by

$$W_a = \int P_e \times dV_a, \quad (8\ c, 10\ b)$$

the calculated actual work is given by

$$\begin{aligned} W_a &= \int P_e \times V_o \times dV_r, \\ \text{or} \quad W_a &= V_o \int P_e \times dV_r. \end{aligned} \quad (8\ d, 10\ c)$$

Since typical vacuoles are of 1×10^{-9} to 1×10^{-12} liters volume (4), absolute work values are of similar orders of magnitude, i.e., 1×10^{-9} to 1×10^{-12} liter atmospheres. Computed values of energy expenditure during enlargement (7) are commensurate with vacuolar volume changes of about 1×10^{-10} liters.

Summary

The means of computation of the coefficient of enlargement of osmotic systems and the work expended in accomplishing such enlargement are presented. Evaluation of these is made for cases which are assumed to be representative of circumstances obtaining with plants at least over a portion of their volume enlargement.

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References

1. Broyer, T. C.: The movement of materials into plants. Part I. Osmosis and the movement of water into plants. — *Bot. Rev.* 13: 1. 1947.
2. — On the theoretical interpretation of turgor pressure. — *Plant. Physiol.* 25: 135. 1950.
3. — An outline of energetics in relation to the movement of materials through a two-phased solution system. — *Plant. Physiol.* 26: 598. 1951.
4. — Further theoretical considerations of modes of expression and factors possibly concerned in the movement of materials through a two-phased solution system. — *Plant. Physiol.* 26: 655. 1951.
5. Burström, H.: Mechanism of cell elongation. — In, *Plant Growth Substances* Ed. by F. Skoog, Univ. of Wisc. Press, Madison, Wisc., 43. 1951.
6. Crafts, A. S., Currier, H. B., & Stocking, C. R.: *Water in the Physiology of Plants.* — Chronica Botanica Co., Waltham, Mass. 1949.
7. Frey-Wyssling, A.: Physiology of cell wall growth. — *Ann. Rev. Physiol.* 1: 169. 1950.
8. Haines, F. M.: The relation between cell dimensions, osmotic pressure and turgor pressure. — *Ann. Bot. n. s.* 14: 385. 1950.
9. — The dynamics of cell expansion by turgor. — *Ann. Bot. n. s.* (a) 15: 219. 1951; (b) 16: 143. 1952.
10. Levitt, J.: Toward a clearer concept of osmotic quantities in plant cells. — *Science* 113: 228. 1951.
11. Lewis, G. N. & Randall, M.: *Thermodynamics and the Free Energy of Chemical Substances.* — McGraw-Hill Bk. Co., N.Y. 1923.
12. Meyer, B. S.: The water relations of plant cells. — *Bot. Rev.* 4: 531. 1938.
13. Moore, W. J.: *Physical Chemistry.* — Prentice-Hall Inc., N.Y. 1950.
14. Suto, I.: On the suction work and elasticity of the plant cell. (In Japanese). — *Bot. and Zool.* 4: 1330. 1936.
15. Tamiya, H.: Zur Theorie der Turgordehnung und über den funktionellen Zusammenhang zwischen den einzelnen osmotischen Zustandgrößen. — *Cytologia* 8: 542. 1938.
16. Thoday, D.: On the water relations of plant cells. — *Ann. Bot. n. s.* 14: 1. 1950.
17. — Turgor pressure and wall pressure — *Ann. Bot. n. s.* 16: 129. 1952.

Two Methods of Fractionating Potato Tuber Proteins and some Preliminary Results with Dormant and Active Tubers

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In a previous paper (5), a preliminary method for extracting and fractionating potato tuber proteins was described. The usefulness of the method was demonstrated by the discovery that tuber halves permitted to sprout at room temperature for 10 days possessed a larger fraction of their crude protein in the water-soluble state (as determined by this method) than did corresponding halves kept at $+2$ to $+5^{\circ}$ C. It was deemed desirable to follow these preliminary results with a more detailed fractionation. Since the methods have also been used to investigate enzyme changes (7) and ion accumulation (6), they will be described here in detail, together with some of the protein changes detected by their utilization. It should be emphasized at the outset, that the purpose of the fractionation procedures is not to obtain pure proteins. On the contrary, the goal is to maintain the proteins in as nearly their normal impure, complex forms as possible. This type of fractionation has already led to extremely important results in the hands of those workers who have investigated the mitochondria and microsomes (2).

Though method 1 has now been completely dropped in favor of method 2, it will be described for three reasons:

- (a) some very interesting results have been obtained with it and will be reported here and in two succeeding papers.
- (b) method 2 is really an improvement of method 1 and is better understood if method 1 is described first.
- (c) some laboratories may not be equipped with a highspeed centrifuge and may therefore wish to use method 1 or some modification of it.

Materials

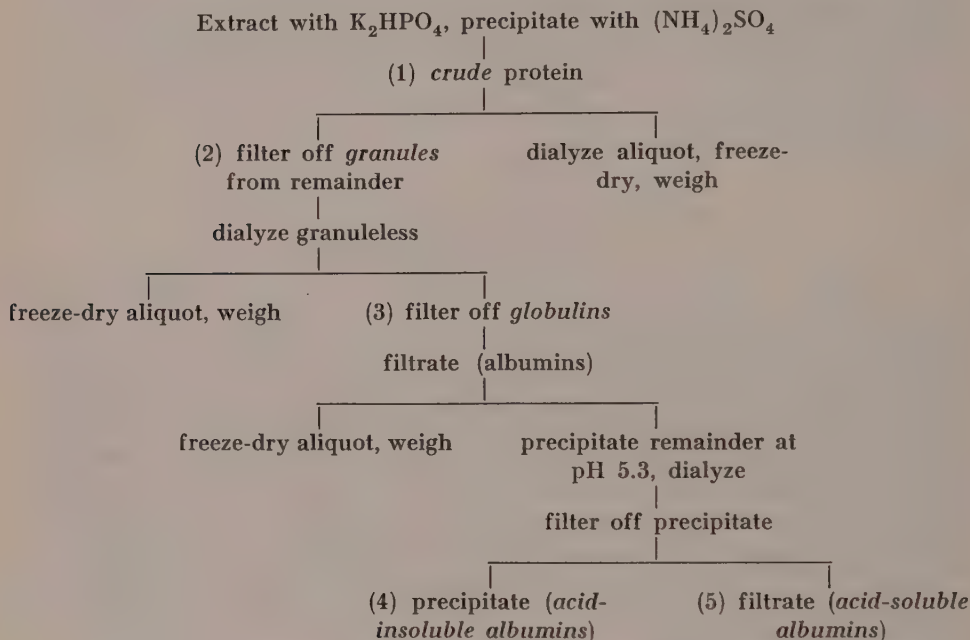
As in previous years, Burbank Russet potatoes were used. Each year, at the end of August or beginning of September, a supply of tubers was obtained for the whole year's work. Thus all the experiments of each year used the tubers of a single clone and this reduced the biological variation to a minimum. The tubers were immediately transferred to a cold room at $+3^{\circ}\text{C}$ ($\pm 1-2^{\circ}$) where they were stored for the whole year or until used. One lot was also stored in a constant temperature room at 26°C ($\pm 1-2^{\circ}\text{C}$) in the dark. On or about the first of each month, two or three medium-sized tubers were halved, one set of halves put in a large covered petri dish at room temperature ($+26^{\circ}\text{C}$) the other set in a similar petri dish in the cold room ($+3^{\circ}\text{C}$). After 10 days both sets were peeled, sliced, freeze-dried, and ground as previously described (5) and kept in a desiccator at $+3^{\circ}\text{C}$ until used. The choice of medium-sized tubers is necessary in view of observations that size of tuber affects time required for sprouting (1).

Methods

Fractionation method 1. Though this method is an extension of the one already described (5), some slight changes were made and it will therefore be described completely. Thirty grams of potato powder were extracted in the cold room with 120 ml. $0.04\text{ M K}_2\text{HPO}_4$. The mash was filtered through No. 1 Whatman paper with suction and was then washed through with 25 ml. H_2O yielding an extract with a pH of 6.5—7.0. Nitrogen analyses of the residue indicated that about 70 per cent of the protein was extracted in this way (assuming that all the residue N was protein N). The extract was then precipitated by saturation with $(\text{NH}_4)_2\text{SO}_4$ plus enough NH_4OH to maintain the pH at about 6.5 (62.5 g. $(\text{NH}_4)_2\text{SO}_4$ plus 2 ml. $0.75\text{ N NH}_4\text{OH}$ per 125 ml. extract). The precipitate was filtered off by using No 50 Whatman paper with suction. About 15 ml. were run through first until the filtrate was clear, then the whole was filtered. The precipitate was then scraped off the filter paper with a spatula and resuspended in water in a graduated centrifuge tube to make exactly 15 ml. 1.5 ml. were pipetted into a flat dialyzing membrane (5) and dialyzed in several changes of water until the dialysate gave no test for SO_4^{2-} with BaCl_2 (4—6 hrs.). This dialyzed portion of the precipitate was then freeze-dried and weighed, enabling calculation of the total »crude protein«. The remaining 13.5 ml. of crude protein was filtered through 2 grams celite deposited on No 50 Whatman paper. This removed all the granules visible under the microscope. The residue was washed through successively with 20 ml. and 10 ml. of $3:1\ 0.1\text{ M K}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$. The combined filtrates were then dialyzed in flat membranes. During this dialysis a precipitate formed. When free of SO_4^{2-} (6—7 hours), this »granuleless« extract was measured, and a 5 ml. aliquot pipetted into an evaporating dish, freeze-dried, and weighed. This enabled calculation of the total »granuleless« components, which when subtracted from the total crude yielded the amount of granules. The remaining dialyzed »granuleless« proteins were then filtered through 3 g. celite deposited on No. 50 Whatman paper and the residue washed through twice with 20 ml. and 10 ml. H_2O . The celite removed the water-insoluble globulins. The filtrate, consisting of the water-soluble albumins, was measured and 15 ml. pipetted into an evaporating dish and freeze-dried. This enabled calculation of total albumins and

of globulins by difference from the total granuleless. Enough $M \text{ KH}_2\text{PO}_4$ was then added to the albumins to make them 0.1 M for KH_2PO_4 , yielding a pH of 5.3 and precipitating some of the albumins. The mixed albumins were then dialyzed in flat membranes until free of phosphate, which was not until the next day. The acid-precipitated albumins were then filtered off by No. 50 Whatman paper; this required two or three filtrations in order to obtain a clear filtrate. Both fractions were then freeze-dried and weighed.

The whole fractionation procedure can be shown schematically as follows:



Some 30 to 50 per cent of the granules and globulins were recoverable by re-suspending them with the celite in $3:1 \text{ } 0.1 \text{ M } \text{K}_2\text{HPO}_4 + \text{KH}_2\text{PO}_4$ and centrifuging off the celite (at about $600 \times \text{G}$) until the supernate was free of diatom cells. This was the method used when enzymatic activities of these fractions were determined. In all enzyme work, aliquots of each fraction were removed and tested as soon as possible, and the rest of the fraction treated according to the above scheme.

There are many disadvantages to method 1. Separation of the fractions was incomplete, partly because some adsorption of the filtrates on the celite must have occurred. Thus the granule and globulin fractions were undoubtedly too large. It is also a distinct disadvantage to have to determine the granules and globulins by difference instead of directly, and to be unable to recover more than 30 to 50 per cent of these fractions. This incomplete recovery was responsible for the use of so large a sample of potato powder (30 g.). Precipitation of the granules by $(\text{NH}_4)_2\text{SO}_4$ has been shown to lead to severe changes (4). The many dialyses lead to losses of enzyme activity (7), and of ions accumulated (6). The 28-hour period required to complete the fractionation process was undoubtedly too long to prevent changes

even at $+3^{\circ}$ C. The use of phosphate buffer for extraction precluded accurate analyses of the fractions for phosphate, since tests showed that even after dialysis some of the added phosphate remained on the proteins. The inability to make more than one run at a time was a distinct drawback. Yet method 1 was able to yield some interesting results (see below and the above two papers). However, as soon as a high-speed centrifuge was available, method 1 was replaced by method 2, which eliminated the disadvantages listed above, as well as several others.

Fractionation method 2. The value of a high-speed centrifuge for obtaining granules (mitochondria and microsomes) has been amply demonstrated by many workers for both animal and plant material (8). This eliminates the $(\text{NH}_4)_2\text{SO}_4$ precipitation which has been shown to alter these particles (4) and the loss of filtrate due to adsorption on the celite that occurs in method 1. Dialysis was eliminated by using a solution of a volatile salt (ammonium acetate) to extract and wash the granules.

The use of ammonium acetate has several other advantages over phosphate. It, of course, prevents any contamination with phosphate and therefore analyses of the fractions for phosphate are possible. Similarly, if the fractions are to be analyzed for metal content, the ammonium acetate can be obtained spectroscopically pure by neutralizing redistilled ammonium hydroxide with redistilled acetic acid. Thus no metal impurities can be added with the salt.

The precipitated and washed fraction was then simply freeze-dried in a desiccator over Al_2O_3 , in this way getting rid of both water and salt. Control lots of dry salt completely disappeared in 24 hours under these conditions. That no more free ammonium acetate remained in the protein samples was shown by transferring the freeze-dried samples to a vacuum oven at 65° C and 15 inches of vacuum for several hours after weight equilibrium had been reached at room temperature. This failed to cause any further decrease in dry weight of the samples. The proteins insoluble at pH 5.3 («acid-insoluble proteins») were next precipitated with acetic acid, centrifuged, washed with 0.2 M ammonium acetate solution, and also freeze-dried without dialysis. The remaining two fractions (globulins and acid-soluble albumins) required $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis, but this one dialysis was rapid (6 to 7 hours) and involved the two most stable protein fractions; at least they remained in the salt-soluble and water-soluble states respectively when freeze-dried. The globulins were separated from the albumins by centrifuging. Thus all five fractions were recovered in their entirety, and no calculations by difference were required. Due to complete recovery, a much smaller sample of potato powder (10 g.) sufficed. The whole fractionation procedure (for two samples) required only 13 to 14 hours from the time of extraction to the time of freeze-drying of the last sample. It was standard procedure to run the two treatments simultaneously so as to eliminate any possible small differences in technique.

The centrifuge used was a Servall SS-2, capable of up to nearly 60,000 xG. It was kept in the cold room ($+3^{\circ}$ C) where all the fractionations were performed. Due to the fact that the case is evacuated and the bearings are water-cooled (the water was circulated through a coil immersed in a mixture of ice and water before being pumped back), the temperature of the samples never rose above $+5^{\circ}$ C at the lower speeds or above $+13^{\circ}$ C at the highest speeds, and even these temperatures were quickly lowered again.

Preliminary tests showed that after centrifuging for 10 minutes at 14,000 xG,

repeated centrifuging at this speed and at higher speeds up to about 30,000 xG failed to bring down any further granules. Consequently, there was apparently no gradation in particle size between the granules thrown down at 14,000 xG («mitochondria») and those obtained at 43,000 xG («microsomes»). No more of the latter were obtained at 52,000 xG than at 43,000 xG.

In the earlier experiments (February and March samples), it was, unfortunately, assumed that filtration through No. 1 Whatman removed all the starch-containing plastids. Observation of the precipitate, however, revealed a smaller white precipitate at the bottom of the larger yellow-brown precipitate. Examination under the microscope showed that this white precipitate consisted of spherical or nearly spherical, highly refractive particles grading in size from obvious starch granules down to particles practically as small as those in the yellow-brown precipitate. These spherical particles stained blue with I_2 in KI, revealing their starch content while the major yellow-brown precipitate stained a bright yellow — characteristic of proteins. From this fact it was concluded that the white precipitate consisted of starch-filled plastids, the yellow-brown precipitate of mitochondria. The plastids were found to be almost completely removed by centrifuging first for 20 minutes at 1000—2000 xG. This failed to precipitate any appreciable amount of the mitochondria and left only enough plastids to form a barely discernible pin-point precipitate at the bottom of the mitochondria as a result of the second centrifuging at 14,000 xG.

Since time was of the essence, the usual method of washing the precipitate by resuspending, recentrifuging and decanting the supernate was avoided. This would also have had the disadvantage of excessively increasing the bulk of the supernate for each successive step. Since the precipitates formed small, compact pellets, not more than 1—3 mm. thick, the procedure adopted was to rinse the pellets twice with one or two mls. of acetate or water (depending on the nature of the pellet). This never dislodged any of the pellets and undoubtedly removed most of the impurities.

The pellets were resuspended in a solution of 0.1 M NH_4 acetate adjusted to pH 8.25 with NH_4OH . By use of a stirring rod with a rubber policeman it was always possible to obtain stable suspensions in this medium. Aliquots of these suspensions could then be used for enzyme determinations. The remainder of each fraction was transferred to a weighing bottle and freeze-dried. In order to prevent breakage of the weighing bottles during this process, it was necessary to tip them so as to expose a large, diagonal surface, at least in the case of the albumins; the other samples contained enough salt to prevent a hard freeze.

The procedure, in detail, was as follows:

1. 10 g. potato powder were extracted with 40 ml. 0.1 M NH_4 acetate adjusted to pH 8.25 by the addition of NH_4OH (1.5 ml. 0.75 N NH_4OH + 10 ml. 7 M NH_4 acetate, diluted to 100 ml.). The mixture was filtered through Whatman No. 1 with suction. This yielded an extract with a pH between 6.5 and 7.0. The residue was reextracted twice with 10 ml. 0.2 M NH_4 acetate, the final extract coming through clear and colorless. All three filtrates were combined.
2. The extract was then centrifuged for 20 minutes at about 1000—2000 xG (the tachometer does not record accurately in this range). The supernate was decanted and centrifuged for 10 minutes at 14,000 xG. The second supernate was again decanted and the pellets rinsed twice with 0.2 M NH_4 acetate. The rinsings were added to the second supernate. The pellets were resuspended in 3 to 5 ml.

- of the above 0.1 M NH_4 acetate solution at pH 8.25, and transferred to tared weighing bottles, in which they were freeze-dried. This yielded the *mitochondria*.
3. The second supernate (plus rinsings) was centrifuged for one hour at 43,000 xG. The third supernate was decanted, the pellets were rinsed twice with 0.2 M NH_4 acetate, and the rinsings added to the supernate. The pellets were resuspended in 3 to 5 ml. of 0.1 M NH_4 acetate at pH 8.25, transferred to a tared weighing bottle, and freeze-dried. This yielded the *microsomes*.
 4. Molar acetic acid was added at the rate of 1 ml. to 10 ml. of the third supernate (plus rinsings). The solution was mixed, then centrifuged for 10 minutes at 14,000 xG. The fourth supernate was decanted, the pellets were rinsed twice with 0.2 M NH_4 acetate and the rinsings added to the supernate. The pellets were then resuspended in 3 to 5 ml. 0.1 M NH_4 acetate at pH 8.25, transferred to a tared weighing bottle, and freeze-dried. This yielded the *acid-insoluble proteins*.
 5. The fourth supernate (plus rinsings) was added to $(\text{NH}_4)_2\text{SO}_4$ at the rate of 2 ml. per g. and thoroughly stirred. The precipitate was centrifuged for 10 minutes at 14,000 xG. The fifth supernate was decanted and discarded. The pellets were quickly rinsed with a little redistilled H_2O , then resuspended in a minimum of redistilled H_2O — a total of about 3 to 5 ml. The suspension was transferred to a flat cellophane membrane and dialyzed for 6 to 7 hours with hourly changes of water, until the dialysate was free of SO_4^{2-} .
 6. The dialyzed protein was then centrifuged for 10 minutes at 14,000 xG. The fifth supernate was decanted, the pellets rinsed twice with redistilled H_2O , and the rinsings added to the supernate. The pellets were then resuspended in 3 to 5 ml. 0.1 M NH_4 acetate at pH 8.25, transferred to a tared weighing bottle and freeze-dried. This yielded the *globulins*.
 7. The fifth supernate was transferred to a tared weighing bottle and freeze-dried. This yielded the *acid-soluble albumins*.

Results

a) *Method 1.* In view of the time-consuming nature of this method and the large sample needed, duplicates could not be conveniently run. The use of tubers of one clone and of the same size, and the comparison of halves of the same tuber reduced biological variability to a minimum. In order to determine whether the standardization of technique was sufficient to yield reproducible results, sixty grams of potato powder were thoroughly mixed, divided into two lots, and fractionated on two days one week apart. These duplicates are compared with two other tubers freeze-dried two weeks earlier (Table 1).

The duplicates show excellent agreement between the crude protein, the granules, the acid-soluble albumins, and the sum of acid-insoluble albumins plus globulins. The latter two fractions, however, vary markedly. This variation was found (in these as well as other experiments) to be dependent on the filtration of the globulins. Sometimes the celite became clogged and filtration proceeded extremely slowly. In such cases, some of the proteins that

Table 1. *Fractions obtained from duplicate samples of potato powder and from similar tubers freeze-dried two weeks earlier. All tubers kept at +26° C from September 17. All values expressed as percents of potato powder.*

Fraction	Dec. 1 tuber (trace of sprouting)	Dec. 1 tuber (large sprouts)	Dec. 17 tubers (large sprouts)	
			1	2
crude protein	2.87	2.36	2.50	2.63
granules62	.40	.45	.47
acid-soluble albumins75	.53	.65	.70
acid-insoluble albumins63	.38	.76	.62
globulins87	1.08	.64	.84
	1.50		1.46	

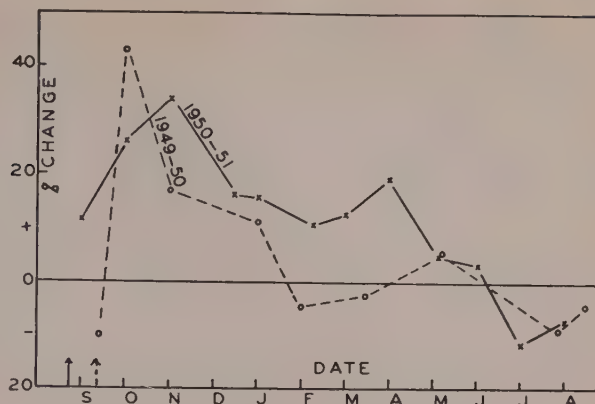
normally passed through were apparently held back with the globulins. This resulted in a larger globulin fraction and a smaller acid-insoluble albumin fraction. The attempt was therefore made to prevent such slow filtration as much as possible. However, in view of these results, the separate values for globulins and acid-insoluble albumins cannot be relied on.

The difference between the December 1 (trace of sprouting) and December 17 tubers are definitely larger (2—8 ×) than the differences between duplicates. But the differences between the two December 1 tubers (trace of sprouting vs. large sprouts) are of the same order as those between the two dates. These differences definitely appear to be related to the degree of sprouting. Thus, both December 1 and December 17 tubers with large sprouts had smaller percents of crude protein, of granules, and of acid-soluble albumins. The decrease in crude protein with sprouting is certainly not fortuitous (Table 4). That the decrease in granules with sprouting is significant was later proved by transferring tubers from +3° to 26° C (Table 6). The decrease in acid-soluble albumins is not so pronounced as the decrease in granules but is consistent in the three sets, and again agrees with the later results (Table 7). There is no significant difference between the sums of the acid-insoluble albumins and the globulins, and the data are insufficient to distinguish between these individual fractions.

When tubers were stored at +3° C over long periods of time and individual tuber halves transferred to +26° C for 10 days, a marked synthesis of crude protein occurred during the fall months of two successive years (fig. 1). That this is a characteristic result is shown by comparing the late fall results for these two years with duplicates during the third year (Table 2).

Since these results are expressed as per cent of dry matter, and since carbohydrates are undoubtedly respired during the 10-day period at +26° C, a slight apparent increase in percent protein might result from this carbohydrate loss.

Figure 1. Percent change in crude protein of potato tuber halves transferred from 3° C to 26° C for 10 days. Abscissa gives date of transfer. Arrows show beginning date of storage at 3° C (broken line 1949, solid line 1950).



How much of a change in carbohydrate would have to occur to account for the above results can be easily calculated. The percent of proteins can be expressed as

$$\frac{x}{x+y+z}, \text{ where } x = \text{grams protein per 100 g potato powder}$$

$$y = \text{grams respirable carbohydrate}$$

$$z = \text{remaining dry matter.}$$

Suppose $x=2.5$, $y=40$, $z=57.5$. Then the percent protein $= \frac{2.5}{100} = 2.5\%$.

In order to obtain an apparent increase in protein (Table 2), due to a decrease in respirable carbohydrate, the following results would be necessary

$\frac{x}{\frac{5}{6}(x+y+z)}$, i.e. the total weight of potato powder would have to be reduced by $16\frac{2}{3}$ g.

If this change in the denominator were due solely to y , it would mean that $\frac{16\frac{2}{3}}{40}$ or more than 40 per cent of the total respirable carbohydrates in the tuber would have to be used up in the 10-day period at +26° C. This would mean that a *non-sprouting* tuber would have to use up all its carbohydrate reserves during storage at +26° C for 26 days!

But it is easy to observe that nothing like such a rapid loss of the tuber's carbohydrates occurs. In fact, after 9 months' storage in the dark at +26° C, the tubers were still alive and still retained ample carbohydrate reserves in spite of the large sprouts formed. Consequently, the above results cannot possibly be explained by carbohydrate loss.

In contrast to the results during the fall, a loss of protein occurred during

Table 2. *Synthesis of crude protein during a 10-day period at +26° C after 2—3 months' storage at +3° in the fall. Values expressed as percent of dry matter of tuber.*

Temperature treatment	Nov. 1949	Dec. 1950	Dec. 1951	
			tuber 1	tuber 2
2—3 months at +3°C	1.20	2.55	2.70	2.98
2—3 months at +3°C followed by 10 days at +26°C	1.40	2.90	3.23	3.61
% increase	17	18	20	21

Table 3. *Loss of crude protein during a 10-day period at +26° C after 9—11 months' storage at +3° C. Values expressed as percent of dry matter of tuber.*

Temperature treatment	July 1950	July 1951	July 1952	
			1	2
9—11 months at +3°C	2.55	2.85	3.47	3.53
9—11 months at +3°C followed by 10 days at +26°C	2.25	2.55	3.17	3.06
% decrease	12	11	9	13

Table 4. *Change in per cent crude protein on storage of tubers at +26° C.*

Date	State of tubers	Crude protein	
		% of potato powder	% decrease
Sept. 17/51	no sprouts	3.52	
Oct. 2	» »	3.08	—12.5
Nov. 1	» »	2.96	—16.
Dec. 1	trace of sprouts	2.87	—19.
Dec. 1	large sprouts	2.36	—33.
Dec. 19	» »	2.24	—36.

10 days at +26° C after 9 to 11 months' storage at +3° C (fig. 1). This was again corroborated by duplicates taken during the third year (Table 3).

That the respiratory loss of carbohydrates does not of itself produce any apparent increase in crude proteins was shown by keeping tubers at +26° C from the time of purchase (Table 4).

Obviously, the loss of crude protein is far more rapid than the loss of carbohydrate. It is very interesting that continuous storage at +26° C should produce a marked, immediate loss of proteins, whereas tubers stored at +3° C for 1 to 3 months show a synthesis of crude protein on transfer to +26° C for 10 days. This is not due to a loss of proteins at +3° C. An initial loss does occur during the first month, similar to the loss at +26° C

Table 5. *Changes in crude protein on storage at +3° C.*

Date	1949—50 crude protein		Date	1950—51 crude protein		Date	1951—52 crude protein	
	o/o of potato powder	o/o change		o/o of potato powder	o/o change		o/o of potato powder	o/o change
Sept. 10	2.50		Aug. 24	2.10		Sept. 17	3.52	
Oct. 1	1.55	—38	Sept. 1	1.95	— 7	Oct. 2	3.08	—12.5
Nov. 1	1.30	—68	Oct. 1	2.40	+14	Nov. 1	3.04	—13.5
			Nov. 1	1.95	— 7	Dec. 13	2.86	—19
Jan. 1	2.40	— 4	Dec. 13	3.80	+40	Jan. 1	2.67	—24
Feb. 1	2.25	—10	Jan. 1	2.35	+12			
			Feb. 1	2.25	+ 7			

(Table 5), but the further continued loss at +26° C is at least retarded at +3° C and, in fact, may be reversed.

The variation in these results is, of course, greater than in the previous cases, since different tubers are compared instead of corresponding halves of individual tubers. In all three years there is a loss of protein during the first few weeks' storage at +3° C. In the first two years the original higher protein content was nearly recovered or even exceeded during successive months. In the third year no such recovery was found, but this may perhaps be related to the much higher original protein content of this year's tubers. It is interesting that in all three years the protein content appears to level off to about the same value by January 1. The 1951—52 protein drop was certainly markedly slowed down as compared with the tubers kept at +26° C. But this slowdown occurred only when sprouting had started in the +26° C lot. During the first 2—6 weeks the protein loss was the same at +3° C as at +26° C.

Thus there are certain generalizations that can be made from three years' results, using method 1.

a) When tubers are transferred to +3° C in September to October, an initial drop in proteins occurs during the first few weeks which is apparently identical with the drop at +26° C. Once sprouting occurs at +26° C, this protein drop is speeded up. The non-sprouting tubers at +3° C show either a slow-down in protein loss or an actual gain, perhaps depending on whether their original crude protein content was high or low. After 2—3 months' storage at +3° C, a 10-day transfer to +26° C results in a 17 to 21 per cent increase in crude protein. After 9 to 12 months at +3° C, a 10-day transfer to +26° C results in a 9 to 13 per cent decrease. At times between these extremes the changes are small and may be either increases or decreases.

Other results with the individual fractions are dealt with in other papers (6), (7).

b) Method 2. The results obtained with this method clearly show that three of the fractions (mitochondria, microsomes, and acid-insoluble proteins) undergo a marked drop during the 10-day period at $+26^{\circ}\text{C}$ (Table 6). The other two fractions (globulins and acid-soluble albumins) undergo no significant change, except that the albumins decreased slightly during May (Table 7). The albumins showed a marked increase at $+3^{\circ}\text{C}$ though none of the other fractions showed any consistent change at $+3^{\circ}\text{C}$ that can be relied on. The apparent decrease in the mitochondria at $+3^{\circ}\text{C}$ is due to the technique used. The first four mitochondria fractions (February and March) contained an undetermined amount of starch-filled plastids (see method above).

c) Comparison of methods 1 and 2. Since method 2 was a development of method 1 with an attempt to improve every step of the procedure, it is not to be expected that the two methods will yield quite comparable results. Since only some 70 per cent of the total protein was extracted by method 1, the extraction procedure adopted in method 2 was designed to increase this yield by a) using more solution per gm. powder, b) extracting three times instead of twice, c) filtering through a »pad» of mash only $\frac{1}{3}$ as thick, due to use of $\frac{1}{3}$ the sample size in the same size funnel. This would reduce adsorption of proteins and clogging of the pores between the mash particles by proteins. d) reduction of filtration time due to the smaller sample. This would also reduce the time for adsorption. That a better extraction is obtained by method 2 is shown in Table 8. Apparently, it is mainly the globulins and acid-soluble albumins that are much more thoroughly extracted by method 2.

Judging from these results, method 2 extracts some 90 per cent of the proteins instead of the 70 per cent obtained by method 1. It is, unfortunately, not possible to check this conclusion directly by nitrogen analyses of the residue since such very small amounts of nitrogen are left in the residue that they may conceivably be due to adsorption of traces of the ammonium acetate and ammonium hydroxide used in extraction, or to formation of slightly soluble salts of these.

The larger granule fraction obtained by method 1 is partly due to inclusion of the small, starch-filled plastids which were centrifuged off in method 2. Obviously, however, the granules obtained by method 1 are far too high to be accounted for in this way, due to retention of some of the other proteins by the celite. The much higher acid-soluble albumin fraction obtained by method 2 can easily be accounted for by the correspondingly higher value of the granule fraction by method 1. In other words, the granule fraction obtained by method 1 is too high because it apparently includes a significant amount of acid-soluble albumins. Thus the sum of granules plus acid-soluble

Table 6. *Mitochondria, microsomes, and acid-insoluble proteins in tubers stored continuously at +3° C and those transferred to +26° C for 10 days. g/100 g potato powder.*

Date freeze-dried	Mitochondria			microsomes			acid-insoluble		
	3°C	26°C	o/o change	3°C	26°C	o/o change	3°C	26°C	o/o change
Feb. 11	1. 0.315	0.187		0.218	0.150		0.884	0.625	
	2. 0.330	0.225		0.230	0.140		0.778	0.626	
av.	0.322	0.206	-36	0.224	0.145	-35	0.829	0.626	-25
Mar. 11	1. 0.150	0.110		0.206	0.143		0.709	0.608	
	2. 0.250	0.175		0.320	0.158		0.758	0.581	
av.	0.200	0.143	-29	0.263	0.151	-43	0.734	0.595	-19
Apr. 11	1. 0.158	0.121		0.235	0.197		0.846	0.747	
	2. 0.171	0.102		0.209	0.156		0.730	0.643	
av.	0.165	0.112	-32	0.217	0.177	-19	0.788	0.695	-12
May 12	1. 0.160	0.170		0.202	0.155		0.800	0.679	
	2. 0.134	0.135		0.170	0.156		0.758	0.722	
av.	0.147	0.153	+4	0.186	0.156	-16	0.779	0.701	-10

Table 7. *Globulins, acid-soluble albumins and total extracted protein in tubers stored continuously at +3° C and those transferred to +26° C for 10 days. g/100 g potato powder.*

Date freeze-dried	globulins			acid-soluble albumins			total extracted proteins		
	3°C	26°C	o/o change	3°C	26°C	o/o change	3°C	26°C	o/o change
Feb. 11	1. 1.090	0.916 *		1.055	1.180		3.562	3.047	
	2. 1.078	1.195		1.195	1.140		3.608	3.326	
av.	1.084	1.056	-3	1.125	1.160	+3	3.585	3.187	-11
Mar. 11	1. 1.255	1.220		1.170	1.084		3.490	3.165	
	2. 1.178	1.230		1.135	1.102		3.641	3.246	
av.	1.217	1.225	+1	1.155	1.093	-5	3.569	3.207	-10
Apr. 11	1. 1.065	0.943		1.262	1.403		3.566	3.411	
	2. 1.190	1.256		1.350	1.305		3.650	3.462	
av.	1.128	1.100	-2	1.306	1.354	+4	3.608	3.436	-5
May 11	1. 1.250	1.305		1.738	1.458		4.150	3.767	
	2. 1.330	1.295		1.600	1.520		3.992	3.828	
av.	1.290	1.300	+1	1.670	1.489	-11	4.071	3.793	-7

* slight loss of sample.

Table 8. *Extraction of protein fractions by methods 1 and 2 on duplicate samples of potato powder. Each series an average of two runs.*

Fraction	Method 1		Method 2	
	% of powder	% of crude	% of powder	% of crude
crude	2.57		3.33	
granules	0.46	18	0.28	8.4
acid-soluble albumins ...	0.68	26.4	1.13	33.7
acid-insoluble albumins ..	0.69	26.8	0.58	17.5
globulins	0.74	28.8	1.35	40.4

albumins (as per cent of crude) is very slightly larger in method 1, due presumably to the small amount of plastids included.

The total of acid-insoluble proteins plus globulins agrees well by the two methods, though distribution among the two fractions does not. But it has already been shown (Table 1) that this distribution varies greatly when method 1 is used, whereas it is remarkably constant when method 2 is used (Table 7).

That method 2 succeeds in separating the acid-insoluble proteins from the globulins (where method 1 fails) is indicated by several facts. (1) The troublesome celite filtrations are avoided. (2) The acid-insoluble proteins are separated before the globulins and before any dialysis can change their properties. (3) The acid-insoluble fraction (method 2) shows a distinct decrease during 10 days at $+26^{\circ}$ C whereas the globulins remain constant. (4) The globulins (by method 2) readily go back into clear solution, whereas the acid-insoluble proteins form an opaque suspension.

Discussion

Fractionation of protoplasmic proteins may have different purposes in the hands of different workers. For the biochemist, the aim is to obtain pure proteins. For the physiologist, the aim should perhaps be to separate the protoplasm into a number of associations of proteins as they exist in the living cell. The two methods described here are attempts to approach the latter goal. Thus in both methods 1 and 2 many precautions are taken to prevent denaturation or at least to reduce it to a minimum — by freeze-drying, by grinding in the dry state and avoiding the injurious action of blending, by performing all the extractions and fractionations at $+3^{\circ}$ C, by reducing the time for these processes to a minimum. Method 2 goes farther in this respect than method 1, by requiring only half the time, by eliminating all except one dialysis, by separating the larger and smaller granules, etc.

That method 1 is useful in detecting changes not otherwise noted was shown by the consistent protein changes it succeeded in revealing during three successive years, by quantitative differences between fractions of tubers in different states of sprouting, and by enzyme differences between corresponding fractions of active and dormant tubers that could not be detected in the crude protein (7).

That method 2 is useful was shown by the marked and consistent decreases in three fractions on transition from the dormant to the active state, and the lack of change in the other two fractions (except a slight one later in the season). That these effects are seasonal is also indicated since the changes decreased with time in storage, i.e. with emergence from the rest period.

It must be emphasized that the methods used are still somewhat arbitrary, method 2 less so than method 1 since the particulates are separated. Consequently, it is not to be expected that results obtained with the two methods will agree completely with one another.

Nevertheless, both methods have shown the following changes associated with sprouting of tubers: a marked decrease in total crude proteins and in the granules, and a slight decrease in the acid-soluble albumins. The inability of method 1 to separate the acid-insoluble albumins consistently from the globulins prevents any comparison of these two fractions by the two methods. Method 1 also appears unable to obtain an adequate separation of the granules from the acid-soluble albumins. What interpretation can be made of these protein changes it is not as yet possible to decide.

Many other experiments have been and will be performed using these methods, and a complete evaluation of their usefulness can be made only after all these results are available. Suffice it to say that the ultimate aim of these methods is to fractionate the protoplasm into its normal constituents in as near their normal states as possible, and to determine what changes these constituents undergo during transition from one physiological state to another.

Summary

Two methods of fractionating the protoplasmic proteins of potato tubers are described, the first based primarily on filtration, the second primarily on highspeed centrifugation. By the first method it was shown that proteins increase about 20 per cent when tuber-halves are transferred from $+3$ to $+26^{\circ}$ C for 10 days during late fall and decrease about 10 per cent during a similar transfer in summer. Tubers kept constantly at $+26^{\circ}$ C showed a steady drop in protein content, which became more rapid as soon as sprouting

was initiated. A similar initial drop occurred during three successive years on transfer to $+3^{\circ}$ C, but after a few weeks either the original protein content was largely regained or at least the drop was markedly slowed up as compared with tubers at $+26^{\circ}$ C. Differences between fractions associated with sprouting are indicated.

By the second method it was shown that conversion of tubers from the dormant to the active state by transfer from $+3^{\circ}$ to $+26^{\circ}$ C for 10 days resulted in a large decrease in mitochondria, microsomes and acid-insoluble proteins, whereas the globulins and acid-soluble albumins remained unchanged. The changes in the first three fractions decreased as the tubers came out of their rest period. These changes associated with sprouting are essentially in agreement with the changes found by the first method.

A comparison of the two methods indicates that method 2 yields a more nearly complete extraction and a better separation of the fractions.

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References

1. Emilsson, B.: Studies on the rest period and dormant period in the potato tuber. — *Acta Agriculturae Suecana* 3: 189. 1949.
2. Green, D. E.: The cyclophorase system. — In *Enzymes and Enzyme Systems, their State in Nature*. Ed. J. T. Edsall. Harvard Univ. Press, Cambridge. 1951.
3. Harman, J. W.: Studies on mitochondria. I. The association of cyclophorase with mitochondria. — *Exp. Cell. Res.* 1: 382. 1950.
4. — — II. The structure of mitochondria in relation to enzymatic activity. *Ibid* 1: 394. 1950.
5. Levitt, J.: The isolation and preliminary fractionation of proteins from dormant and growing potato tubers. — *Plant Physiol.* 26: 59. 1951.
6. Long, William G. & J. Levitt. — In press.
7. Todd, Glenn W. — In press.
8. Umbreit, W. W., Burris, R. H., and Stauffer, J. F.: *Manometric techniques and related methods for the study of tissue metabolism*. — Burgess. 1945.

Bioassay, Purification, and Properties of a Growth Factor from Coconut

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The recent extensive use of coconut milk to promote growth of tissues and organs of higher plants *in vitro* derives from the successful application of the material by van Overbeek et al. (9) to the culture of *Datura* embryos which were removed from the ovaries at early stages of development. Subsequent work by these investigators (10), Caplin and Steward (4), Steward and Caplin (11), Nickell (8), Ball (2), and Morel and Wetmore (7), has shown that coconut extracts exert a marked growth-promoting effect on a variety of plant tissues, and Howell (6) has found a marked effect of coconut milk on the development of excised pea epicotyls *in vitro*.

Some evidence has been obtained that more than one active ingredient may be present in the coconut milk (R. Siu, private communication). The factor required for growth of *Datura* embryos has been found to be present also in malt extract (Blakeslee and Satina, 3), yeast extract, *Datura* embryo extract, almond meal and wheat germ (van Overbeek et al., 10). The factor which stimulates the growth of carrot tissue has been found also in extracts of milky-stage corn kernels (Steward, 4), and in carrot leaves (Wiggans, 13).

Some attempts have been made to isolate the active materials. Van Overbeek, Haagen-Smit and Siu (10) obtained about 170 times increased activity per unit dry weight by repeated 78 per cent alcohol extraction, precipitation

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with lead acetate, and precipitation by acidifying the alcoholic filtrate with H_2SO_4 to pH 3. In a recent paper Steward, et al. (12) report that the material active on carrot tissue is a heat stable, water soluble, organic compound. However, as far as we are aware no evidence has been published on the further purification or chemical nature of the growth factor.

Work in this laboratory has shown that coconut milk is active in promoting growth of a variety of plant tissues in vitro. In order to evaluate its effects, it was necessary to obtain it free from auxin, sugars, and other possible active constituents. The material has therefore been concentrated and purified on the basis of its activity in promoting growth of tobacco or carrot tissues under conditions where indoleacetic acid (IAA) and carbohydrates are ineffective.

This paper reports the methods of bio-assay and the procedures used in preparing a concentrate of the active constituent of coconut meat some 4350 times more active than the starting material on a fresh weight basis.

Experimental

Materials

The coconuts used were obtained in the local market. Early experiments showed that most of the activity was in the meat which therefore was used instead of the milk as source material.

In the early experiments tobacco callus obtained from stem tissue of *Nicotiana tabacum*, Wis. No. 38, and subcultured through several transfers was used for assaying. In later tests, the callus formed on segments of carrot root tissue cultured in vitro, (*Daucus carota*, variety Red Cored Chantennay, grown in southern Wisconsin or carrots of unknown source obtained in local markets) was used as stock material.

Assay Procedure

Root segments about $1.5 \times 1.5 \times 1$ cm. taken from the middle third of the root are sterilized by rubbing off with 95 per cent alcohol, soaking in 0.1 per cent HgCl_2 for 20 min. and rinsing 3 times in sterile distilled H_2O . These stock pieces contain mostly phloem tissue but also some cambial and xylem tissue.

They are cultured on modified White's medium. It contains sucrose, 20 g./l., and the following constituents in mg./l.: KNO_3 80, MgSO_4 72, $\text{Ca}(\text{NO}_3)_2$ 144, KCl 65, KH_2PO_4 38, KI 0.75, MnSO_4 6.5, $\text{Fe}_2(\text{SO}_4)_3$ 2.5, ZnSO_4 2.7, H_3BO_3 1.6, thiamin 0.1, nicotinic acid 0.5, pyridoxine 0.5, and glycine 2.0. Cysteine hydrochloride, 1 p.p.m., is also included as recommended by Gautheret (5)

for continuous culture of carrot tissues. The carrot segments develop a fairly uniform surface layer of callus which is 1 to 2 mm. thick after a growth period of about 6 weeks. After this time the callus layer is removed, cut into small fragments (approximately $2 \times 2 \times 0.5$ mm.), and these fragments are transferred to vials containing 1 ml. of nutrient medium with the material to be tested added as a supplement.

The test media are prepared as follows: The material to be tested is added to 15 ml. double strength, modified White's medium, the mixture made up to 30 ml., the pH adjusted to 4.5 with HCl or NaOH, and 270 mg. of agar added to make a 0.9 per cent gel. The solutions are heated and 1 ml. is pipetted into each of 24 shell vials (15×45 mm.) mounted in a rack. The vials are plugged with cotton and autoclaved at 15 lbs. for 15 min. Carrot fragments are transferred aseptically to the surface of the medium after it has cooled, one fragment being planted in each vial. The fragments are distributed at random through all the controls and samples so that variation in the callus cannot account for differences in growth between treatments. The cultures are grown in a physiological dark room at 24° C. and 85—90 per cent relative humidity. In this manner the cultures can be kept for 2 weeks without evidence of deficiencies or serious desiccation of the medium. Doubling or tripling the nutrient volume has no effect on the growth.

Since the pieces may undergo some shrinkage during and subsequent to transplanting, measurements of their size are made after 12 to 24 hrs., when they have reached fairly constant size and have become firmly attached to the agar surface. The cross-sectional areas are estimated in terms of the product of the longest diameter and the diameter perpendicular to it. The measurements are made by inverting the vials under a dissecting microscope and observing the fragments in focus on an ocular micrometer. The vials are marked and record of measurements kept on each individual fragment. The sizes of the fragments at successive stages of growth are expressed as a percentage of their original sizes.

As shown by typical growth curves in Figure 1, the controls increase in size only slightly whereas tissue fragments supplied with optimal concentrations of a potent extract may increase significantly within 4 to 5 days and continue to grow rapidly for a 2-week period. Final measurements are usually made after a 9 or 10-day growth period. The measurement of cross-sectional areas rather than volumes tends to minimize the estimates of the actual increase but is preferable to measurements of weight in that the pieces can be measured repeatedly and individually with a minimum of labor.

Potencies of various fractions have been expressed in terms of an arbitrary unit defined as the activity contained in the water-soluble material from 1 g. of fresh coconut meat. Maximum growth is elicited by a concentration of

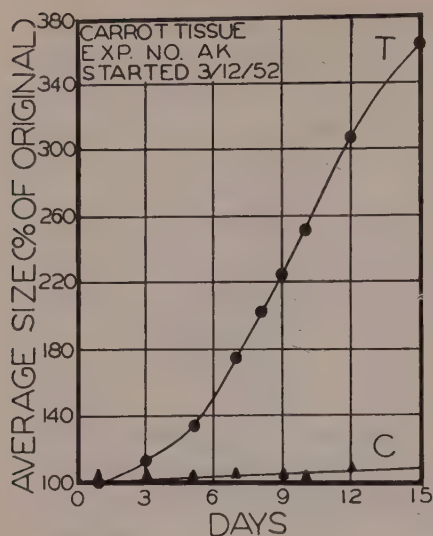


Figure 1. Increase in size (cross-sectional areas) of carrot callus fragments with time. Data are averages of 24 cultures. C, controls; T, supplied with coconut factor concentrate (G-1, 15 units/100 ml.).

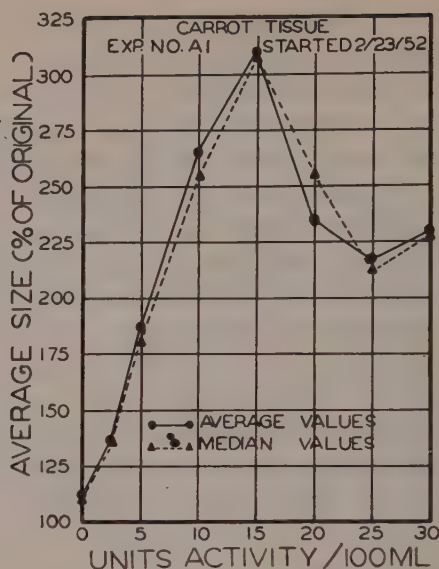


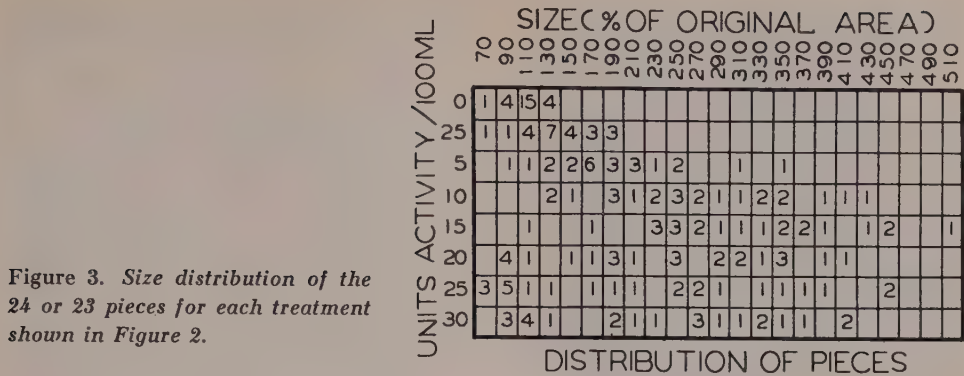
Figure 2. Effect of concentration of coconut concentrate (G-1) on the growth of carrot callus. Final measurements after a 10-day growth period.

about 15 units per 100 ml. of test medium, whereas higher concentrations cause inhibition (Figure 2). Since it was anticipated that various coconuts would show considerable variation in activity, a single large batch of a potent concentrate has been used as the actual working standard. The preparation of this concentrate (G-1) is described below.

Fractions to be assayed are set up at concentrations estimated to fall in the range 5—15 units per 100 ml. of test medium. The G-1 standard is also included with each assay at a level of 15 units per 100 ml. of medium. The potency of each unknown sample is estimated by comparing its growth-stimulating effect (percentage increase in area of the test pieces minus that of the untreated controls) with that of the standard, as follows:

$$\frac{\text{Sample, \% increase}}{\text{G-1, \% increase}} \times \frac{15}{\text{g. sample per 100 ml. test medium}} = \text{Units per g. of sample}$$

The final measurements on the 24 individual pieces used in each test are plotted in size-distribution diagram (Figure 3). These show that whereas the distribution in size increase of the control pieces is within a narrow range (70—130 per cent), the distribution for the pieces supplied with active



extract is spread over relatively wide ranges, so that a large number of pieces are required to obtain accurate quantitative measurements. This spread was even larger in the early experiments, but was narrowed considerably by the use of large stock pieces of the size designated above. The callus on these large pieces is more uniform and thus provides more uniform test material. It may be seen, furthermore, that with super-optimal concentrations of the extract the decrease in growth is due to a shift in the individual size distribution, so that even though some pieces grow very large, there is a larger number of pieces that fail to grow or increase only slightly. In general it is clear that although there may be considerable variation in the quantitative measurements, there is no difficulty in determining whether or not the preparations are active even when they are tested in lower or higher than optimal concentrations. In fact, as shown by the photograph in Figure 4, the difference in size between treated and control tissues after a 9 or 10-day growth period is obvious even from visual inspection.

In early experiments with tobacco callus the control pieces grew to varying extents between about 120—180 per cent of the initial size. Hence, it was sometimes difficult to determine whether the increases over the controls were significant. It was for this reason that the assay procedure was shifted to the use of carrot callus. However, as shown by the curves in Figure 5, fairly appreciable and consistent increases with increasing amounts of active extracts were also obtained in tobacco callus cultures even when the controls increased to 160 per cent of their original size.

Specificity of the Assay

Numerous tests have been carried out to determine the interfering action (inhibition or stimulation of growth) by various chemicals. The following

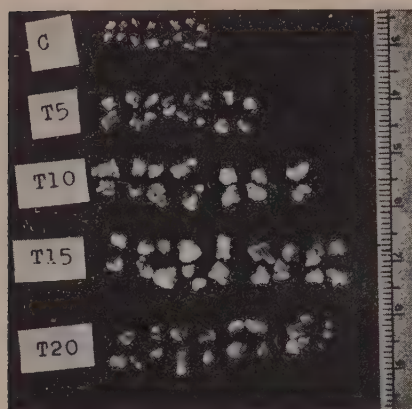


Figure 4. Photograph of representative pieces supplied with increasing concentrations of coconut concentrate (G-1) from 0 to 20 units/100 ml. after a 10-day growth period. C, control; T5, T10, T15, and T20, supplied with 5, 10, 15, and 20 units of activity per 100 ml. respectively.

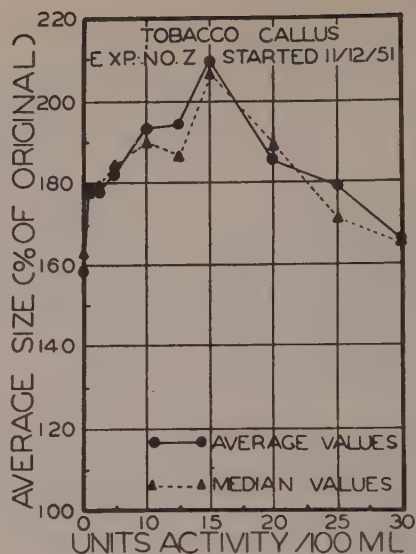


Figure 5. Effect of concentration of coconut concentrate (G-1) on the growth of tobacco callus. Final measurements after a 10-day growth period.

compounds showed no effect or were inhibitory when they were added alone in the concentrations indicated to the basic nutrient medium. *Sugars*: sucrose (3 %), glucose (5 %), arabinose (1, 5, 25 p.p.m.). *Amino compounds*: arginine hydrochloride (1, 10 p.p.m.), cysteine (1 p.p.m.), L-tryptophan (1, 10, 100 p.p.m.), L-asparagine (1, 10 p.p.m.). *Purines and pyrimidines*: xanthine (20, 60 p.p.m.), guanine (20, 60 p.p.m.), hypoxanthine (50 p.p.m.), adenosine (50 p.p.m.), adenylic acid (50 p.p.m.), adenosine triphosphate (50 p.p.m.), uridine (20 p.p.m.), uracil (10, 64 p.p.m.), hydrolysed sodium nucleate (20 p.p.m.), hydrolysed deoxyribonucleic acid (20 p.p.m.). *Miscellaneous organic compounds*: sebacic acid (0.1, 1, 10 p.p.m.), 2,4-dichlorophenoxyacetic acid (0.1, 1, 7 p.p.m.), indoleacetic acid (1, 10 p.p.m.), folic acid (0.5, 5 p.p.m.), inositol (100 p.p.m.), leucovorin (0.004, 0.04 p.p.m.). The synthetic citrovorum factor was kindly supplied by The Lederle Laboratories. *Inorganic compounds*: sodium chloride (167, 334, 500 p.p.m.), ammonium nitrate (1, 5, 10, 25 p.p.m.), silver chloride (saturated aqueous solution). *Natural extracts*: casein hydrolysate (1 %), yeast extract (1 %).

The following compounds and extracts were active to the extent indicated: Adenine was very slightly active at 5 p.p.m. but inactive at 0.5, 1, 10, 20, or 50 p.p.m. Biotin was very slightly active at 1 and 2 p.p.m. An extract of

barley seed in the milky stage, an extract from Jack Pine seed, and an extract of the vascular tissue of tobacco all duplicated the stimulatory effect of the coconut meat extract.

When added to media containing 15 units of G-1 per 100 ml., the following compounds gave the results indicated: Biotin at 1 p.p.m. showed slight inhibition of the coconut effect; adenine at 5 p.p.m. gave a slight increase; 2,4-dichlorophenoxyacetic acid had little effect at 1 p.p.m. but was strongly inhibitory at 7 p.p.m.; sodium chloride had no effect at 500 p.p.m. but at 3300 p.p.m. inhibited the coconut effect slightly. Indoleacetic acid at 1 p.p.m. gave a considerable increase in combination with highly purified extracts such as the Neuberg precipitate (prepared as described below).

Effects of certain other factors have also been tested. A shift in pH of the control medium from 4.5 to 6.2 had no significant effect on the growth of the fragments, but in media containing coconut extract, the growth rate was slightly higher at pH 4.5 than at 3.5, 5.5, or 6.5. In tests with tobacco callus, variation in the agar concentration from 0.7 to 1.5 % had no effect on the growth rates.

The following compounds were tested on larger (5×5×5 mm.) pieces of callus from carrot tissue on 50 ml. of medium in 125 ml. Erlenmeyer flasks. None had any effect at the concentrations shown when added alone (*i.e.*, without coconut factor) to the basic nutrient medium. L-histidine (10, 100 p.p.m.), L-glutamic acid (50 p.p.m.), pyridoxamine hydrochloride (5 p.p.m.), allantoin (50 p.p.m.), D-ribose (10, 100 p.p.m.), L-galactose (100 p.p.m.), L-aspartic acid (10, 50, 100, 500 p.p.m.), L-arabinose (100 p.p.m.), D-lyxose (100 p.p.m.), maltose (100 p.p.m.), D-mannose (100 p.p.m.), L-sorbose (100 p.p.m.), L-xylose (100 p.p.m.), glucose-1-PO₄ (300 p.p.m.), glutathione (1, 10, 50 p.p.m.), thymine (10, 40 p.p.m.).

Purification Procedures. — Extraction and Preparation of a Preliminary Concentrate

In a typical experiment, the milk from 19 nuts was drained and discarded, and the meat (5400 g.) was homogenized with distilled water (2 ml./g. meat). A Waring Blendor was used for these homogenations. The homogenate was filtered through cheesecloth, and the residue reextracted once as before. The bulk of the fat was removed from the milky extract by passage through a cream-separator. The aqueous phase was concentrated in vacuo to ca. 1 liter, heated to 100° at pH 4.5 (natural pH) for 30 minutes, and the denatured proteins removed by filtration with the use of a filter aid (»celite-545», Johns Manville Co.). The precipitate was washed well with water (ca. 300 ml.), and the filtrate and washings were combined and concentrated to

900 ml. Ethanol was added to a final concentration of 75 per cent, and the ethanolic solution was refrigerated at 4° for 12 hours. Clarification was accomplished by filtration through a filter aid. After concentrating to ca. 250 ml., the filtrate was extracted three times with ether to remove residual lipoidal material. Subsequent concentration in vacuo yielded a dark yellow syrup (159 g.) which was used as stock supply for further investigations. This product was designated fraction G-1 (1.0 g. G-1 equivalent to 34 g. fresh meat). Activity: 34 units/g.

Neuberg Precipitation. G-1 (28.0 g.) was dissolved in 80 ml. of water and the pH was adjusted to the phenolphthalein end point (external indicator) with a saturated solution of sodium carbonate. Mercuric acetate (1 ml. of 25 per cent aqueous solution) was added dropwise. A cream colored precipitate appeared. Sodium carbonate was added until the phenolphthalein end point was just reached, and then the mercuric acetate addition was repeated. Alternate additions of sodium carbonate and mercuric acetate were continued until an orange precipitate appeared. To this mixture (100 ml.) was added 50 ml. of ethanol, and the solution was refrigerated for 12 hours to insure complete precipitation. After centrifugation, the supernatant was discarded and the precipitate washed once with cold 80 per cent ethanol and recentrifuged. The Neuberg precipitate was suspended in water and hydrogen sulfide was passed through the suspension for 20 minutes. The precipitated mercuric sulfide was removed by centrifugation, and the supernatant was concentrated to dryness in vacuo.

Dry weight: 1.37 g. Activity: 332 units/g. Yield: 48 per cent.

Electrodialysis. The Neuberg precipitate, 125 mg., was further purified by electrodialysis. The apparatus employed was similar to the one described by Albanese (1) except that five compartments were used, 10 ml. volume each. Platinum wires were used as the electrodes. Distilled water was added to the electrode compartments, and 2 drops each of 1.0 N HCl and KOH were added to the anode and cathode compartments, respectively. An aqueous solution (pH 3.6) of the Neuberg precipitate (125 mg.) was added to the central compartment (compartment no. 2) and distilled water added to a total volume of 10 ml. Compartments 1 and 3, on the anode and cathode sides of the central compartment, respectively, were filled with distilled water. »Visking» dialysis membrane (Dialysis tubing, 19 mm., cut open. Supplied by Geo. T. Walker and Co.) was used to separate the compartments. Direct current, 115 volts, was used, and the amperage was followed with a milliammeter. The initial current of 1 ma. rose steadily to a maximum of 65 ma. in 45 minutes. Within 50 minutes the current had dropped to 42 ma., and at this time the pH values of compartments 1, 2, and 3 were 3.1, 3.9, and 9.3

respectively. The electrodialysis was stopped and the solutions in each compartment evaporated to dryness in vacuo.

Compartment Number	Dry Weight	Activity, units/g.	Yield, %
1	3.5 mg.	0	—
2	81.1 mg.	15.3	3
3	20.0 mg.	2005	97

Electrodialysis of the Neuberg precipitate (135 mg.) was also carried out after adjusting the sample pH to 10.3 with 0.10 *N* KOH. The current reached a maximum of 130 ma. in 35 minutes. Within 55 minutes the current had dropped to 45 ma., and at this time, the pH values of compartments 1, 2, and 3 were 2.5, 3.5, and 10.5 respectively. The dialysis was stopped and the solutions in each compartment were evaporated to dryness in vacuo.

Compartment Number	Dry Weight	Activity, units/g.	Yield %
1	6 mg.	1018	14
2	62 mg.	615	85
3	32 mg.	693	50

Paper Chromatography of Dialyzed Bases. Experiments designed to reveal optimum conditions for separation were worked out on material from compartment 3 from the dialysis run at pH 3.6. Descending paper chromatograms were run for 18 hours in a 150×800 mm. battery jar from stainless steel troughs. Sample spots were placed on the paper, dried in air and equilibrated with the system for three hours before the solvent was added to the trough. Strip paper No. 613, 0.5 inch wide, supplied by Eaton and Dikeman was used as received. The samples, 20 μ g., were applied to the paper in volumes of 10 μ l. per spot. Four solvent systems were investigated. System A composed of *n*-butanol equilibrated against water, gave no resolution. System B, composed of 75 ml. *n*-butanol, 25 ml. propionic acid, and 30 ml. of water gave resolution of three ninhydrin positive spots. Unfortunately, the solvent front moved very slowly. It was thought that this slow movement might be due to esterification, and so a third solvent system, C, was used which was identical with system B, except that *t*-butanol was substituted for *n*-butanol. Although the movement of the solvent front was a little more rapid with this third system, the resolution was much poorer. System D, composed of 75 ml. of methyl-ethyl-ketone, 25 ml. of propionic acid and 30 ml. of water gave resolution of 4 ninhydrin positive spots having R_F values of 0.35, 0.41, 0.57, and 0.68, respectively.

When solvent system D was used with material from compartment 3 of the pH 10.3 dialysis above, four ninhydrin-positive spots with R_F values of 0.26, 0.35, 0.57, and 0.71, respectively, were observed.

A second strip was tested for guanido compounds by a modified Sakaguchi test. The paper was sprayed successively with 10 per cent NaOH, 0.2 per cent α -naphthol, and NaOBr (0.2 g. Br_2 in 10 ml. 5 per cent NaOH). A 3 minute drying time was allowed between each spraying. Development of an orange-red spot (R_F 0.35) indicated the presence of one or several guanido compounds.

Stability of the Growth Factor. An active preparation (G-1, 2.5 g.) was dissolved in 15 ml. of water, divided into three equal portions, and treated as follows:

- a. The pH was adjusted to 11.2 with 3.0 ml. of 3 *N* NaOH and the resulting alkaline solution was heated at 100° for two hours.
- b. Ten ml. of conc. HNO_3 was added and the acidic solution was heated at 100° for 3 hours.
- c. Ten ml. of 5 per cent HCl was added and the solution evaporated to dryness under vacuum at 50° bath temperature. The last traces of HCl were removed by repeated dilution and concentration.

The activity was destroyed in each case.

Further Studies on the Properties of the Growth Factor

1. *Attempted liquid-liquid extraction.* An aqueous solution of preparation G-1 was adjusted to pH 11 and extracted with *n*-butanol in a continuous extractor for 24 hours (preliminary batch-wise technique seemed to indicate slight extraction at this pH). Subsequent evaporation and assay of both the aqueous and organic phases showed all of the activity in the former.

2. *Lead precipitation.* Treatment of samples of G-1 with neutral lead acetate, basic lead acetate, and basic lead acetate plus ammonia, and subsequent removal of the lead as the sulfide, showed no activity in the precipitate or filtrate. These data seem to point to the destruction or splitting of the activity, or to the introduction of an inhibitor, i.e. lead ions. These precipitation studies were abandoned in favor of the Neuberg precipitation and subsequent electrodialysis.

3. *Adsorption methods.* Attempts were made to find a suitable adsorbent. »Darco G-60» charcoal, Malinckrodt silicic acid, talcum powder, Merck's chromatographic alumina, silica gel, and five different lot grades of »Nuchar» charcoal were used in these adsorption studies. The activity was held by one of the »Nuchar» lots, but was not held by any of the other adsorbents. All attempts to remove the activity from the »Nuchar» sample failed. Thus,

ethanol, ethanol-HCl, pyridine, glacial acetic acid, and concentrated ammonium hydroxide were all ineffective in liberating the activity.

4. *Ion-exchange studies.* Several ion-exchange studies were carried out on fraction G-1 employing both strong and weak anion and cation exchange resins. In all cases, aqueous solutions (pH 5.0) of G-1 were put on the columns. Amberlite IRC-50 (H form) and IR-4B (OH form), representing the weak cation and anion resins, respectively, did not hold the activity. Dowex-50 (H form), Amberlite IR-100 and 120 (H forms), strong cation exchange resins, and Amberlite IRA-400 (OH form), a strong anion exchanger, all held the activity so tenaciously that successful elution of the factor was never accomplished. The ion exchange studies were therefore abandoned.

5. *Preparation of derivatives.* Since the chemical properties of the growth factor seemed to indicate a basic or amphoteric compound, attempts were made to prepare common amine derivatives. This work was done with the G-1 fraction. Thus, preparation of a picrate, picrolonate, Reinecate, diliturate, and helianthate was attempted. The crude derivatives produced contained only a small portion of the total activity and were, therefore, not further purified.

Discussion

The bioassay method used to follow the active material through the various fractionations has been complicated by the presence of inhibitory substances. Thus, although six of the eight preparations similar to G-1 which have been made to date proved to be potent, the other two showed very slight activity, and only at the highest dilutions tested. Increasing concentrations of these two preparations resulted in progressively less activity. Fortunately, the inhibitory materials were largely removed in later purification steps, for example, during the mercury precipitation. These circumstances have several times resulted in apparent yields of activity greatly in excess of 100 per cent, where certain purification procedures have been carried out. Such was probably the case for example in the electrodialysis experiment at pH 10.3, where the products obtained showed 150 per cent of the activity of the starting material. It is possible that some inhibitors remain even in the most highly purified fractions, since it has been consistently observed that no amount of these concentrates will produce as great a response as G-1 or similar preparations, even though their potencies in terms of units of activity per gram are much higher. The discrepancy in maximum activity between the G-1 fraction and the more highly purified preparations is at least partially overcome by the inclusion of indoleacetic acid (0.1—1 mg./l) in the nutrient medium. The concentrate G-1 itself, however, is relatively free from auxin

activity as its effectiveness is also increased to a small extent in the presence of added indoleacetic acid.

Despite variations in the inhibitor and active factor content of different batches of coconuts, the preparation of preliminary concentrates of the type of G-1 has been almost always successful. The largest batch, involving the use of 100 nuts, happened to be one of the few apparently inactive preparations, but even this yielded an active Neuberg precipitate. In fact, the Neuberg precipitation method has proved to be a most useful purification procedure. Its utility was nearly overlooked because of the fact that at first HCl was added before removing mercury as the sulfide. Whenever this was done, dark-colored, inactive products resulted, whereas Neuberg precipitates decomposed in neutral solution gave very satisfactory products.

The factor may be generally present in plants, but its activity may be masked by high inhibitor concentrations. For example, during the course of the present investigation, yeast extract, which is completely inactive in the above carrot test, yielded an active product after partial purification.

The active factor is characterized by high water solubility, non-volatility, and insolubility in such organic solvents as ether or butanol at any pH between about 1 and 12. Since it is held on both strong acid and strong base ion exchangers, and migrates mainly toward the cathode, the active substance is probably amphoteric and may well contain a strongly basic grouping. Destruction of the activity by ashing, strong acids, and alkalies, and by a strong oxidizing agent constitutes good evidence that the factor is an organic substance. No loss of activity has been observed as a result of autoclaving at neutral pH, or by exposure to ordinary laboratory conditions for limited periods of time. The G-1 concentrate, a honey-like material, has been stored at 4° for nine months without apparent loss of activity.

Although the factor has been concentrated some 4000 times as compared to the fresh coconut meat used as the source material, the final product, as judged by paper chromatography, is still far from homogeneous. Whether any of the ninhydrin- or guanido-reacting substances observed in the paper chromatograms is identical with the active substance has not yet been determined.

Summary

A bioassay and a procedure for the purification of a growth factor from coconuts have been worked out. The purification involved extraction of the meat with water, removal of proteins by heat and alcohol denaturation, and separation of the fat. Precipitation of the factor by the method of Neuberg, followed by electrodialysis yielded an active basic fraction which was shown

by paper chromatography to contain at least four ninhydrin-positive compounds, one of which gave a positive Sakaguchi test for guanido compounds.

Evidence to date indicates that the growth factor from coconuts is a heat stabile, acid- and alkali-labile, non-volatile, water-soluble organic compound. Since the factor moves to the cathode from an aqueous solution during electrodialysis, it seems to have basic properties.

A concentrate of the active constituent of coconut meat has been prepared which is 4350 times more active on a fresh weight basis than the starting material.

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References

1. Albanese, A. A.: An electrolytic method for the determination of the basic amino acids in proteins. — J. Biol. Chem. 134: 467. 1940.
2. Ball, E.: Development in sterile culture of stem tips and subjacent regions of *Tropaeolum majus* L. and *Lupinus albus* L. — Amer. Jour. Bot. 33: 301. 1948.
3. Blakeslee, A. F. & Satina, S.: New hybrids from incompatible crosses of *Datura* through culture of excised embryos on malt media. — Science 99: 331. 1944.
4. Caplin, S. M. & Steward, F. C.: Effect of coconut milk on the growth of explants from carrot root. — Science 108: 655. 1948.
5. Gautheret, R. J.: Manuel Technique de Culture des Tissus Végétaux. — p. 52, Masson et Cie., Paris 1942.
6. Howell, R. H.: A study of the influence of roots on shoot growth. — Ph. D. Thesis, University of Wisconsin, 1952.
7. Morel, G. & Wetmore, R. H.: Tissue culture of monocotyledons. — Amer. Jour. Bot. 38: 138. 1951.
8. Nickell, L. G.: Effect of coconut milk on the growth in vitro of plant virus tumor tissue. — Bot. Gaz. 112: 225. 1950.
9. van Overbeek, J., Conklin, Marie E., & Blakeslee, A. F.: Factors in coconut milk essential for growth and development of very young *Datura* embryos. — Science 94: 350. 1941.
10. van Overbeek, J., Siu, R., & Haagen-Smit, A. J.: Factors affecting the growth of *Datura* embryos in vitro. — Amer. Jour. Bot. 31: 219. 1944.
11. Steward, F. C., & Caplin, S. M.: A tissue culture from potato tuber: The synergistic action of 2,4-D and of coconut milk. — Science 113: 518. 1951.
12. Steward, F. C., Caplin, S. M., & Millar, F. K.: Investigations on growth and metabolism of plant cells. I. New Techniques for the investigations of metabolism, nutrition, and growth of undifferentiated cells. — Ann. Bot. 16: 58. 1952.
13. Wiggans, S. C.: Studies on the growth of tissues of the carrot root *in vitro*. — Ph. D. thesis, University of Wisconsin, 1951.

Factors Controlling Meristematic Activity in Excised Roots. I. Experiments Showing the Operation of Internal Factors

By

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Introduction

Previous work has shown that substitution of ferric citrate or ferric sodium ethylenediamine tetra-acetate for the ferric sulphate of White's medium allows excised tomato roots to continue growth for longer periods (Street, McGonagle, and McGregor, 1952). Study of root growth in media containing these iron complexes showed, however, that increase in dry weight continued after the main axis had ceased to increase in length. Therefore the dry weight increase at this stage arose primarily from the growth of lateral roots. The present paper provides further evidence that under certain conditions the initial meristem does cease growth in medium which is still supporting active growth of the more recently initiated lateral meristems. Experiments are also described showing that lateral root growth is influenced by the main axis meristem and that, in turn, the activity of the main axis meristem is influenced by the rest of the root. Such internal factors, rather than composition or 'staling' of the culture medium are considered to limit the duration of activity of the main axis meristem when excised tomato roots are grown under the conditions described.

Experimental

Culture technique

The general culture technique, composition of the medium and methods of measuring root growth have been already described (Boll and Street 1951;

Street, McGonagle, and Lowe 1951). The clone of excised roots of *Lycopersicum esculentum*, Mill (Best-of-All) used in previous work has again been employed.

Growth in large volumes of culture medium

Previous experiments in which iron complexes have been supplied have involved the use of 50 ml. culture medium per 10 mm. inoculum (Street, McGonagle and McGregor 1952). It was therefore decided to compare the growth of excised roots in larger volumes of medium containing ferric citrate, with growth in corresponding volumes of standard culture medium in order to see how far the activity of the main axis meristem is controlled by non-availability or other changes in medium composition resulting from root growth.

Experiment No. 1 compared the growth in standard culture medium ('S') and in this medium modified by replacing the ferric sulphate by ferric citrate at a concentration equivalent to $\times 2$ the standard iron concentration ('C $\times 2$ '). Each medium was represented by 4 cultures in 500 ml. and 4 cultures in 1,000 ml. using as culture vessels 1,000 ml. and 2,000 ml. Pyrex flasks. The 500 ml. cultures were harvested after 20 days' and the 1,000 ml. cultures after 28 days' incubation. The results are shown in Table 1. The individual values for length of main axis were variable within each treatment

Table 1. *Growth of excised tomato roots (Best-of-All) in standard medium (S) and in this medium modified by replacing the ferric sulphate by ferric citrate to give $\times 2$ the standard iron concentration (C $\times 2$)*

Period of culture (days)	Volume of medium. (ml.)	Medium	Root No.	Length of main axis (mm.)	Dry weight (mg.)
20	500	S	1	242	45.6
			2	254	40.9
			3	174	38.7
			4	224	32.4
		C $\times 2$	1	223	41.4
			2	308	77.8
			3	228	66.8
			4	302	92.4
28	1000	S	1	191	68.7
			2	277	86.7
			3	122	58.2
			4	241	97.6
		C $\times 2$	1	219	196.6
			2	299	199.4
			3	263	154.4
			4	331	197.7

and the results for all four treatments give means within the limits 200—300 mm. There is high variability in the values for main axis length and no marked difference between treatments. By contrast, highly significant differences between treatments are obtained for mean dry weight per root. The greater dry weights recorded with the medium ('C×2') are due to the greater growth of laterals in this medium, associated with the maintenance of iron availability.

Comparison of the values for length of main axis in Table 1 with those reported from earlier experiments using large volumes of medium 'S' (Street, McGonagle, and Lowe 1951) confirms that maximum values are of the order 200—300 mm. in both cases. These experiments support the view that the growth in length of the main axis of the excised tomato root cannot be prolonged beyond a certain value by preventing 'staling' of the culture medium.

Growth in intermittently renewed culture medium

Two possible explanations of the cessation of growth of the main axis before that of the lateral axes in Experiment No. 1 are: —

(i) Changes occur in the culture medium as a result of root growth and render it toxic but this toxicity takes time to manifest itself. Each meristem therefore has a span of activity determined by the intensity of 'staling'. It could be postulated that the operative 'staling' product is slowly accumulated by each meristem and has to reach a critical internal concentration before it stops meristematic activity.

(ii) Each meristem becomes progressively more susceptible to inhibition by 'staling' factors. This postulates an internal change but regards this as reacting with an external change in the culture medium.

Both these explanations require the further postulate that the 'staling' effect is exerted equally over the range of 'staling' intensity covered by the treatments in Exp. No. 1. If the intensity of 'staling' could be reduced to a very low level without any significant increase in main axis length the explanations set out above would become very improbable. Experiment No. 2 was designed with this end in view.

Experiment No. 2 employed as culture vessels 1,000 ml. Pyrex Flasks, fitted with Pyrex siphons and charged with 500 ml. culture medium. Tips were inoculated into 22 flasks, 11 containing culture medium 'S' and 11 containing culture medium 'C×2'. In half the culture flasks the medium was not renewed during the incubation period. From the remaining flasks the culture medium was drawn off via the siphons, under aseptic conditions on the 10th, 14th, 17th, 19th, and 21st day of incubation and on each occasion

Table 2. *Effect of intermittent renewal of the medium on the growth of excised tomato roots (Best-of-All) in media 'S' and 'C×2'. Incubation at $26 \pm 0.5^\circ$ C. Period of culture 23 days. (Experiment No. 2)*

Medium	Total volume involved per culture (ml.)	Root No.	Length of main axis (mm.)	Dry weight (mg.)
'S'	500	1	214	33.1
		2	204	26.0
		3	228	44.0
		4	225	31.7
		5	235	30.0
	3000	6	255	68.0
		7	290	82.8
		8	260	63.3
		9	217	68.0
		10	263	78.9
		11	243	50.3
'C×2'	500	12	245	64.8
		13	233	61.7
		14	213	82.4
		15	288	75.2
	3000	16	260	60.6
		17	225	44.6
		18	182	49.0
		19	220	66.2
		20	209	54.3
		21	202	65.8

replaced by 500 ml. of fresh culture medium. All cultures were harvested after 23 days' incubation. The results are shown in Table 2. Only when employing medium 'S' did renewal of the culture medium enhance significantly the values for root dry weight. The values for length of main axis were not increased by renewal of the medium. The results strongly support the view that the duration of activity of the main axis meristem in these experiments is not limited by 'staling' factors.

Growth in constantly renewed culture medium

The techniques employed in Exp. No. 1 and 2 did not enable us to prove that the main axis had ceased to increase in length before the time of harvesting the roots, although frequent examination of the cultures strongly indicated that this was the case. Furthermore the main apices were, in most cases, distorted in a manner previously observed in small volume cultures which were known to have ceased growth. An apparatus (Apparatus A, figure 1, I) has now been devised which ensures adequate aeration of the culture medium allows it to be renewed either intermittently (*flush method*) or continuously (*drip method*), and makes possible daily determination of

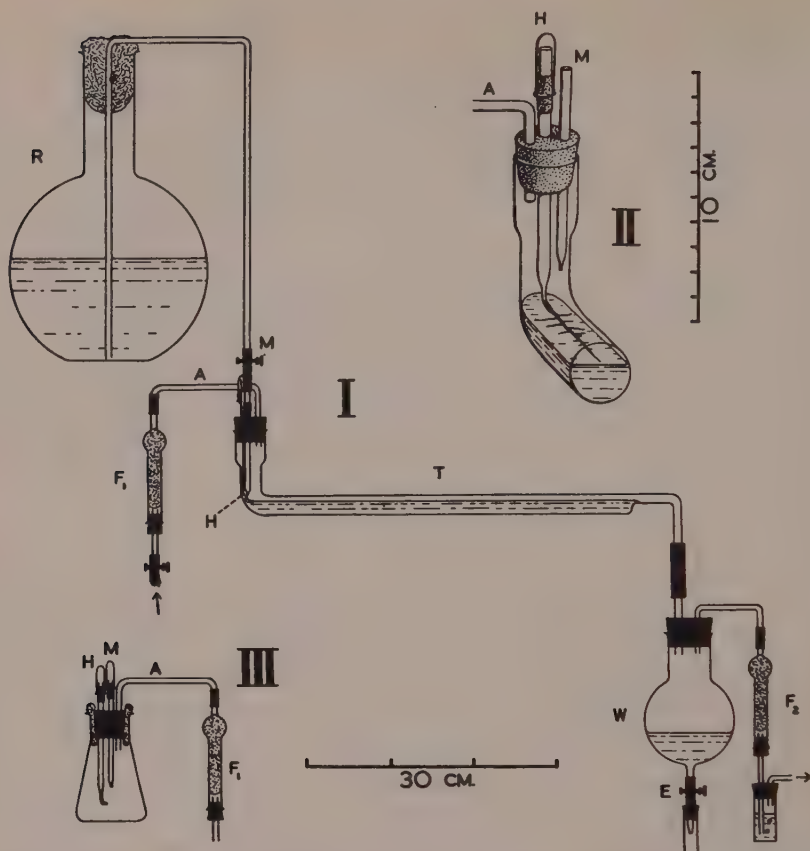


Figure 1. Apparatus A. I. Complete apparatus; II. View of part of the growth tube showing the attachment of the root in its holder; III. Rubber bung, carrying root holder, medium feed tube and air inlet tube, shown in its carrier flask. 30 cm. scale shown for I and III; 10 cm. scale shown for II. R=reservoir of sterile culture medium, fitted with siphon tube; M=culture medium feed tube; A=air inlet tube; H=root holder; T=culture tube; W=graduated effluent trap; E=exit from which effluent medium can be withdrawn from W; F_1 and F_2 =air filters packed with non-absorbent cotton wool.

the length of the main axis of the root and of the pH of the effluent culture medium.

This apparatus is constructed of Pyrex glass and of previously autoclaved rubber tubing. The apparatus is sterilised by autoclaving separately the following components: — (i) the reservoir R containing culture medium and with the opening of the siphon closed by a tightly-fitting sealed glass tube; (ii) the culture tube T, one opening closed by a cotton-wool plug and the other by the graduated effluent trap W with filter F_2 attached; (iii) the rubber bung fixed in its carrier flask (Figure 1, III) and carrying the root holder H, the culture medium feed-tube M and the

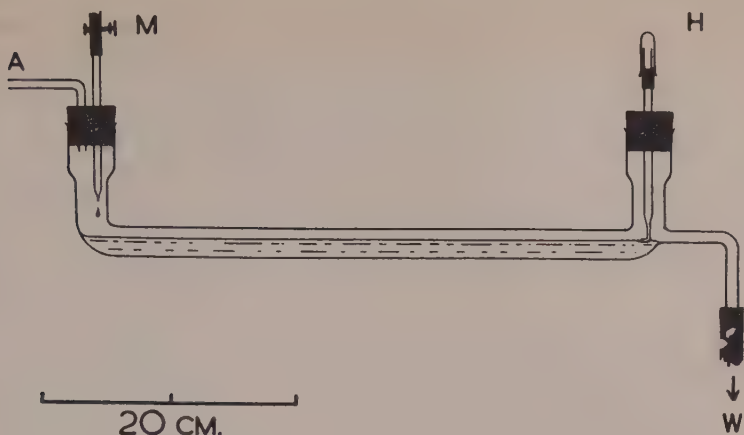


Figure 2. Culture tube used in Apparatus B. Key as in Fig. 1.

air inlet tube A with filter F_1 attached. The carrier flask is charged aseptically with 100 ml. sterile culture medium. A 10–15 mm. root tip is excised from a 7-day sector culture and fixed in the holder H in a sterile inoculating room. This tip then remains immersed in the culture medium contained in the carrier flask until the complete apparatus is assembled in a temperature-controlled room operating at $26.5 \pm 0.5^\circ$ C. Assembly of the complete apparatus involves the following sequence of operations: fixing the culture tube T in a horizontal position; charging this tube with 100 ml. sterile culture medium; removal of the cotton-wool plug and insertion of the rubber stopper and its tubes in the open end of the culture tube; adjustment of the level of the root holder H so that the root tip is just below the surface of the culture medium (Figure 1, II); connecting M to the siphon of the reservoir R; starting of the flow of medium from R, if necessary by gentle suction applied at the outlet from air-filter F_2 ; and finally commencement of the positive pressure air-flow through F_1 and A. The air-flow, generated by an electric blower, is purified by passing through wash-bottles containing, in order, sodium hydroxide solution, dilute sulphuric acid, and distilled water and is then led via A either above (Figure 1, I, II) or below the surface of the culture medium in the growth tube. The rate of air-flow is controlled by screw-clips and estimated by the rate of bubbling at the trap beyond F_2 . New culture medium enters the growth tube at M either (a) twice daily until on each occasion a given volume of medium has collected in W (flush-method) or (b) by a continuous and carefully regulated slow rate of dripping from a specially constructed orifice below M (drip-method). Effluent medium is drawn off at E either once or twice daily and its pH determined. The reservoir R has either 1,000 ml. or 5,000 ml. capacity and is renewed during the incubation period.

A modified form of the apparatus (Figure 2, Apparatus B) has also been used. In this the culture medium flows counter to the direction of root growth so that new medium first meets the main axis meristem and then flows over to the older parts of the root to enter the effluent trap (W) by an outlet near to the holder (H).

Table 3. *Growth of excised tomato roots (Best-of-All) in media 'S' and 'C×2' using Apparatus A and Apparatus B*

Medium	Apparatus	Method of solution renewal: flush (F) or drip (D)	Length of main axis (mm.)
'S'	A	F	230 188 193
		D	294 149
	B	D	146 155
'C×2'	A	F	241 275
		D	231 274

Results obtained by both the '*flush*' and the '*drip*' methods of culture medium renewal are shown in Table 3.

The root inocula grew for variable periods and the values for length of main axis were of the same order as those recorded in Tables 1 and 2. The method of culture medium renewal and the medium used (medium 'S' or 'C×2') did not influence the values obtained for main axis length. When growth ceased, the main axis apices usually showed a typical distortion. Measurements of the pH of the effluent medium showed that with both the '*flush*' and '*drip*' methods of renewal 'staling' of the medium is virtually eliminated. These preliminary results with apparatus A and B have therefore provided further evidence that, when 'staling' is eliminated, the growth of the main axis is limited by other factors.

The effect of excision of the apical 10 mm. upon the length of main axis generated in a given volume of culture medium

The possibility that the duration of activity of the apical meristem was being determined by the composition of the culture medium rather than by internal factors was now considered. It was visualised that a direct and continuous accumulation of some medium constituent might occur at the meristem and that when this reached a critical toxic concentration it inhibited meristematic activity. Alternatively the medium composition might indirectly cause accumulation by the meristem of some toxic metabolite.

Possible effects on the meristem of excising the root apex are: —

(i) removal of an influence exerted on the meristem by the older parts of the root;

(ii) exposure of a cut surface from which, at least temporarily, active exudation of cell materials and particularly xylem sap could take place (White 1938);

(iii) formation at the cut surface of a wound hormone.

Experiments No. 3, 4, and 5 were undertaken to determine the main axis lengths generated by excised as against non-excised meristems grown under conditions of uniform 'staling' or where, if anything, the 'staling' conditions favoured the non-excised meristems.

Experiment No. 3 employed 100 cultures in 50 ml. medium. 20 cultures were harvested after 7 days; 20 cultures after 14 days; 20 cultures after 21 days; 20 cultures had the main axis tip (10 mm.) excised on the 7th day and were harvested after 14 days; 20 cultures had the main axis tip excised on the 7th and again on the 14th day and were harvested after 21 days. At each harvest pH values and root dry weights (Street, McGonagle and Lowe 1951) were determined and main axis lengths measured. Where the main axis tip had been excised, the values for main axis length are the sums of the root 'pieces' generated by the main apex per culture.

Experiment No. 4 was carried out as described above except that in the 'excised' 21-day cultures the tip was only excised on the 7th day.

Experiment No. 5 employed 40 cultures in 100 ml. medium. On the 9th day of culture, the roots were transferred aseptically to new flasks of medium and on the 16th day the medium was removed aseptically from the culture flasks and replaced by 100 ml. of new medium. Each culture was therefore supplied with 300 ml. of medium. Half the cultures had their tips excised on the 9th and again on the 16th day of incubation. All cultures were harvested on the 23rd day.

Medium 'C×2' was used in these experiments to prolong the period over which 'staling' did not completely inhibit growth. From consideration of the volume of medium per culture, dry weight production per culture and pH values at harvest, it is clear that the intensity of 'staling' occurring in Experiment No. 5 was lower than in Experiments No. 3 and 4.

The results of these experiments are shown in Table 4. Excision of the main apex prolongs its activity. The sum of the main axis 'pieces' in the 'excised' cultures significantly exceeds the values for main axis length in the corresponding 'non'excised' cultures although dry weight and pH determinations showed that 'staling' was equally or more intense in the 'excised' cultures. Only by postulating excretion of accumulated toxic materials from the cut surface of the excised apex can the prolongation of meristem activity by excision be considered compatible with the hypothesis that the primary factor limiting meristem function is the composition of the culture medium. Explanation of the prolongation of meristem activity as due to wound hormone formation at excision requires that this hormone exert a 'regenerating' effect on the apical meristem itself. The explanation which seems most probable, and which is capable of further investigation, is that the pro-

Table 4. *Influence of excision of the main axis tip upon increase in length of main axis and increase in dry weight. Medium 'C×2'*

Exp. No.	Volume of medium ml.	Period of culture in days	Treatment	Dry wt. per root (mg.)	Increase in main axis length (mm.)	Results ¹
3	50	7		1.37	63.5±2.24 (20)	
		14		8.64	(a) 99.2±4.29 (18)	b >> a
			tip excised after 7 days	9.36	(b) 115.6±4.51 (18)	
		21		16.6	(a) 120.4±5.47 (18)	b >> a
			tip excised after 7 and after 14 days	16.5	(b) 141.0±5.48 (20)	
4	50	7		1.64	65.5±2.88 (19)	
		14		9.3	(a) 108.1±2.43 (18)	b >>> a
			tip excised after 7 days	9.8	(b) 124.5±2.36 (18)	
		21		15.2	(a) 116.1±7.17 (18)	b >>> a
			tip excised after 7 days	18.8	(b) 147.4±4.46 (18)	
5	100 ml. renewed after 9 days and again after 16 days from commencement of experiment	23		27.5	(a) 161.8±6.8	b >>> a
			tip excised after 9 days and after 16 days	26.1	(b) 209±10.0	

¹ >> difference significant at P=0.02

>>> , , , P=0.01

longation of activity by excision is due to removal of an influence exerted on the meristem by the older parts of the main axis or its associated laterals or both.

Effects of the main axis tip and of other parts of the root on the growth of lateral meristems

Observations of the rates of lateral growth in sector cultures (Street, McGonagle, and Lowe 1951) and of the growth of laterals consequent upon cessation of the activity of the main axis meristem in tip cultures grown under conditions preventing 'staling', suggested that whilst active, the main axis meristem exerts an inhibitory effect on lateral growth. This effect is most marked on the young laterals nearest to the apex; such laterals were described by White (1943) as lacking full individuality or 'dominance'. Quantitative evidence of such an effect has now been obtained (Exp. No. 6).

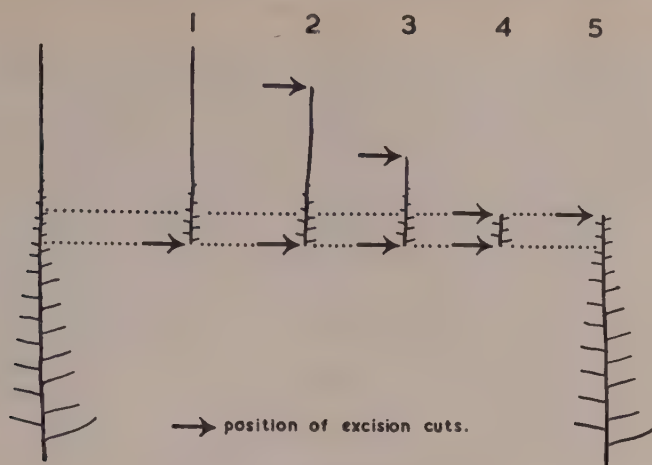


Figure 3. Diagram showing the types of sector used in Experiment No. 6. Positions of excision cuts shown and sector types numbered 1 to 5.

Experiment No. 6. From a batch of 7-day tip cultures selected for uniformity, sector cultures of five types were established (Figure 3). In types 1, 2, and 3 the sector laterals whose growth is to be studied are the five oldest laterals, in type 5 they are the five youngest laterals. These sector laterals were each 3—5 mm. in length at the beginning of the culture period. Type 2 sectors differ from type 1 in that the apical 10 mm of the main axis has been excised. Type 3 sectors differ from type 2 in bearing only half as great a length of main axis above the youngest sector lateral. 18 cultures of each type were incubated for 7 days in medium ('C×2'). This medium was chosen so that 'staling' would interfere as little as possible with growth. pH determinations showed an equal intensity of 'staling' in types 1, 2, 3, and 4 but a somewhat higher intensity in the type of 5 cultures. Growth of the sectors was assessed by summing the lengths of the 5 sector laterals of each culture at harvest. The results are presented in Table 5. The greater length of the sector laterals in type 4 cultures than in types 1, 2, and 3 indicates that both the main apex and the main axis bearing more recently initiated lateral meristems, assert an inhibitory effect on the growth of the sector laterals. The similar values for length of sector laterals in types 2 and 3 cultures suggests that it is the meristems of the younger laterals rather than the main axis itself which exerts the inhibitory effect since the type 3 sectors differ from type 2 in lacking a length of main axis devoid of visible lateral initials. The main axis bearing laterals older than the sector laterals

Table 5. *Influence of the main axis meristem and other parts of the root on the growth of lateral roots. Sectors of the clone (Best-of-All) grown in medium 'C×2' (Experiment No. 6)*

Type of sector	pH of medium at harvest	Sum of length of the 5 sector laterals (mm.) after 7 days culture	No. of replicates	Results ¹
1	6.20	100.8±8.1	18	$\begin{cases} 1 \ll 2 \\ 2=3 \end{cases}$
2	6.21	125.0±6.2	18	$\begin{cases} 3 \ll 4 \\ 4=5 \end{cases}$
3	6.01	125.6±8.4	18	$\begin{cases} 1 \ll 3 \\ 1 \lll 4 \end{cases}$
4	6.24	153.5±6.6	17	$\begin{cases} 1 \ll 5 \\ 2 \lll 4 \end{cases}$
5	6.49	136.0±11.5	18	3=5

¹ < difference significant at P=0.1

<< , , , P=0.02

<<< , , , P=0.01

(type 5 cultures) has little effect on sector lateral development particularly when it is remembered that the type 5 cultures show more intense 'staling' than any other type.

Summary

1. An apparatus is described which enables excised roots to be grown aseptically in constantly renewed culture medium and permits daily measurements of the increase in length of the main axis.

2. When excised tomato roots are grown in constantly renewed culture medium, or in large volumes of culture medium, the main axis meristem only functions for a limited time and ceases activity whilst the more recently initiated lateral roots are growing rapidly.

3. The duration of activity of the main axis meristem can be prolonged by excision and the hypothesis is tentatively advanced that this is due to removal of an influence exerted on the meristem by the main axis and its associated laterals.

4. Evidence is presented that the main axis meristem and younger parts of the main axis bearing lateral root initials exert a depressing effect on the growth rate of young lateral roots.

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References

- Boll, W. G. & Street, H. E.: Studies on the growth of excised roots. I. The stimulatory effect of molybdenum and copper on the growth of excised tomato roots. — *New. Phytol.* 50: 52. 1951.
- Street, H. E., McGonagle, M. P., & Lowe, J. S.: Observations on the 'staling' of White's medium by excised tomato roots. — *Physiol. Plant.* 4: 592. 1951.
- , — & McGregor, S. M.: Observations on the 'staling' of White's medium by excised tomato roots. II. Iron availability. — *Physiol. Plant.* 5: 248. 1952.
- White, P. R.: 'Root pressure' — an unappreciated force in sap movement. — *Amer. J. Bot.* 25: 223. 1938.
- A Handbook of Plant Tissue Culture. — The Ronald Press Co. New York, 1943.

Biotin und *p*-Aminobenzoessäure als Wachstumsstoffe für frisch isolierte *Clostridium*-Formen

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Im hiesigen Institut befindet sich eine grosse Kollektion von *Clostridium*-Stämmen, welche von Richard (1948, 1950) aus den verschiedensten Materialien wie Erde und Abwasser, grünen Pflanzen und Grassilage, buttersaurer Kartoffel-, Mais-, Roggen- und Kastanienmaische, geblähtem Emmentaler- und Schmelzkäse, Kuh- und Schafkot usw. isoliert worden sind. Nach Pasteurisation der Materialien oder ihrer wässerigen Suspensionen wurden dabei in gewissen Fällen *direkt* anaerob verschlossene Strichkulturen auf Glucose-Fleischwasser- oder Glucose-Hefewasser-Agar angelegt. In der Regel aber wurden *zuerst Anreicherungskulturen* in Magermilch, Glucose-Magermilch, Maismaische, Grünerbsmaische, Glucose-Bouillon oder der stickstofffreien Nährlösung nach Winogradsky (1902) hergestellt. An Hand der Ergebnisse zahlreicher Versuche, die so isolierten Bakterienstämme mit den in der Literatur beschriebenen *Clostridium*-Arten zu identifizieren, zeigt Richard (1948, 1950), dass sie durchaus den allgemeinen Charakter von *Cl. butyricum* Prazmowski (vgl. Bergey's Manual 1948, S. 770) aufweisen. Unter diesen Stämmen finden sich einerseits typische Buttersäurebakterien (beispielsweise Stamm 208), welche nebst Kohlendioxyd und Wasserstoff hauptsächlich Buttersäure und Essigsäure bilden, anderseits Butylbakterien (beispielsweise Stamm 174), welche nebst den erwähnten Gasen und Säuren beträchtliche Mengen n-Butylalkohol und Isopropylalkohol bzw. Aceton erzeugen (Wikén 1951; Wikén, Richard und Aebi 1952).

Snell und Williams (1939) wiesen nach, dass ein Stamm von *Cl. butylicum* (Nr. 6015 von der »Amer. Type Culture Collection«) durch Zusatz von Biotin zu kräf-

tigem Wachstum in synthetischen Substraten (Asparagin, Glucose, Mineralsalze) gebracht werden konnte. Peterson, McDaniel und McCoy (1940) stellten fest, dass 7 *Clostridium*-Arten, darunter *Cl. butylicum* und *Cl. sporogenes*, nur einen Zusatz von Biotin brauchen, um in einer synthetischen Nährlösung (Asparagin, Glucose, Mineralsalze) wachsen zu können, während 20 Arten, darunter *Cl. acetobutylicum*, *Cl. botulinum*, *Cl. welchii* und *Cl. histolyticum*, unter den gleichen Bedingungen keine Vermehrung zeigten. Lampen und Peterson (1941) fanden, dass 3 Stämme von *Cl. acetobutylicum* (unter diesen Nr. 862 von der »Amer. Type Culture Collection«) ein synthetisches Substrat (Asparagin oder Asparagin+Ammonsulfat, Glucose, Mineralsalze) assimilieren konnten, wenn dasselbe gleichzeitig Biotin und *p*-Aminobenzoessäure enthielt, während diese Wuchsstoffe, einzeln zugesetzt, kein Wachstum der untersuchten Stämme ermöglichten. In diesem Zusammenhang sei erwähnt, dass Rubbo und Gillespie (1940), welche die Interferenz zwischen *p*-Aminobenzoessäure und Sulfanilamid bei 9 Stämmen von *Cl. acetobutylicum* (u.a. Nr. 862) untersuchten, zur Auffassung kamen, dass die geprüften Stämme für ihr Wachstum in synthetischer Nährlösung (Asparagin, Glucose, Mineralsalze) nur einen Zusatz von *p*-Aminobenzoessäure forderten. Lampen und Peterson (1943) prüften 20 *Clostridium*-Stämme auf ihren Bedarf an Biotin und *p*-Aminobenzoessäure. Sämtliche liessen sich erst nach Zusatz von Biotin in synthetischem Substrat (Ammonphosphat+Ammonsulfat, Glucose, Mineralsalze) züchten. Die Stämme von *Cl. acetobutylicum* konnten die Nährlösung nur ausnutzen, wenn dieselbe gleichzeitig mit Biotin und *p*-Aminobenzoessäure versetzt war. Zum gleichen physiologischen Typus wie *Cl. acetobutylicum* gehörten *Cl. felsineum* (Nr. 41) und einer der geprüften Stämme (Nr. 28) von *Cl. butylicum*, während 6 Stämme der letzten Art sowie *Cl. sporogenes* und *Cl. tetani* (Nr. 459 bzw. Nr. 457 von der »Amer. Type Culture Collection«) nur einen Zusatz von Biotin forderten. Reyes-Teodoro und Mickelson (1945) untersuchten 3 *Clostridium*-Stämme, welche keine augenfälligen proteolytischen, dagegen kräftige saccharolytische Wirkungen aufwiesen und ferner auf Melasse-Substraten grosse Mengen von *n*-Butylalkohol und Aceton nebst kleinen Mengen von Essigsäure und Buttersäure bildeten. In synthetischer Nährlösung (Ammonsulfat, Glucose, Mineralsalze) zeigten 2 Stämme maximales Wachstum nach Zusatz von Biotin allein, während der dritte Stamm zu maximaler Entwicklung eine Kombination von Biotin und *p*-Aminobenzoessäure forderte. Es handelte sich um atypische Stämme von *Cl. acetobutylicum* insofern als sie Stärke nur langsam zu vergären vermochten. Perlman (1948) hat die Wirkung des D-Desthiobiotins und DL-O-Heterobiotins (DL-Oxybiotin) auf »normale« und »degenerierte« Zellen einer Anzahl *Clostridium*-Arten mit derjenigen des D-Biotins verglichen. Die »Degeneration« der Bakterien wurde dabei durch wiederholte Ueberimpfung auf das zur Gewinnung des Impfmateri als verwendete Substrat ohne Pasteurisation erzeugt. Die »degenerierten« Zellen von *Cl. pasteurianum*, *Cl. butylicum*, *Cl. beijerinckii* und *Cl. acetobutylicum* zeigten ein verhältnismässig gutes Wachstum in der Biotin-freien synthetischen Nährlösung (Ammonphosphat+Ammonacetat, Glucose, Mineralsalze), während die »normalen« Zellen kein (*Cl. butylicum*, *Cl. acetobutylicum*) oder nur geringes Wachstum (*Cl. pasteurianum*, *Cl. beijerinckii*) aufwiesen. Bei *Cl. acetobutylicum* und *Cl. beijerinckii* sowie bei je einem Stamm von *Cl. butylicum* und *Cl. pasteurianum* übte Desthiobiotin auf die »normalen« Zellen approximativ die gleiche Wuchsstoffwirkung aus wie Biotin, während es bei je einem weiteren Stamm der letzten zwei Arten und *Cl. felsineum* Biotin nicht ersetzen konnte. Oxybiotin hatte einen verhältnismässig kräftigen fördernden Einfluss auf die Vermehrung der »normalen« Zellen der beiden Stämme von *Cl. bu-*

tylicum, dagegen nur eine geringe wachstumsstimulierende Wirkung auf *Cl. acetobutylicum*, *Cl. beijerinckii* und die beiden Stämme von *Cl. pasteurianum*. Für *Cl. felsineum* war Oxybiotin inaktiv. In diesen Prüfungen enthielten die für *Cl. acetobutylicum* und *Cl. felsineum* verwendeten Substrate 50 µg *p*-Aminobenzoesäure pro ml.

Im Hinblick auf die Ergebnisse der erwähnten Untersuchungen und in Anbetracht der Tatsache, dass wir beabsichtigten, den Verlauf und die Endprodukte der Buttersäure- und Butylgärungen in synthetischen Substraten quantitativ zu ermitteln (Wikén, Richard und Aebi 1952), schien es uns von Interesse, den Bedarf der frisch isolierten *Clostridium*-Stämme an (+)-Biotin und *p*-Aminobenzoesäure zu untersuchen.

Methodik

Die Wachstumsversuche wurden in starkwandigen Reagenzgläsern ohne Rand von 160 mm Länge und 14 mm lichter Weite durchgeführt. Diese wurden nach gründlichem Waschen mit heisser Sodalösung, Spülen mit heissem Leitungswasser und dest. Wasser, Erhitzen mit dest. Wasser im Autoklaven bei 120° C während 15 Minuten und nochmaligem Spülen mit dest. Wasser mit Pfropfen aus chemisch reiner Baumwolle verschlossen und bei 160° C während 1 Stunde im Trockensterilisator erhitzt.

Die Kulturröhrchen enthielten in sämtlichen Versuchen je 9 ml Nährlösung. Folgende Substrate kamen zur Verwendung:

Nährlösung I.

Dieses synthetische Substrat basiert bezüglich seiner Zusammensetzung einerseits auf den Angaben von Block und Bolling (1947, S. 303) über die Verteilung der Aminosäuren in einem Caseinhydrolysat, anderseits auf den Angaben von Möller (1939) über eine zur Züchtung von Milchsäurebakterien geeignete synthetische Nährlösung. Es wird aus den folgenden Teillösungen zusammengesetzt:

Lös. 1:

L(—)-Cystein-hydrochlorid	0,20 g
Dest. Wasser	ad 20 ml

Lös. 2:

Glycocoll	0,05 g
DL-Alanin	0,20 g
L(+)-Arginin	0,20 g
DL-Asparaginsäure	0,20 g
DL-Phenylalanin	0,20 g
L(—)-Tryptophan	0,20 g
DL-Valin	0,20 g
L(+)-Glutaminsäure	0,50 g
DL-Leucin	0,50 g
Dest. Wasser	ad 400 ml

Lös. 3:

DL-Histidin-monohydrochlorid . .	0,20 g
DL-Isoleucin	0,20 g
L(+)-Lysin-dihydrochlorid	0,20 g
DL-Methionin	0,20 g
L(—)-Oxyprolin	0,20 g
DL-Prolin	0,20 g
L(—)-Tyrosin	0,20 g
Dest. Wasser	ad 300 ml

Lös. 4:

DL-Cystin	0,05 g
DL-Serin	0,20 g
DL-Threonin	0,20 g
Dest. Wasser	ad 100 ml

Lös. 5:			KH_2PO_4	1,36 g
D(+)-Glucose	20,00 g		$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	0,42 g
Dest. Wasser	ad 80 ml		$\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$	0,025 g
Lös. 6:			$\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$	0,013 g
$\text{CH}_3 \cdot \text{COONH}_4$	6,42 g		Dest. Wasser	ad 100 ml
$\text{Na}_2\text{HPO}_4 \cdot 2 \text{H}_2\text{O}$	1,78 g			

Die Aminosäuren wurden einzeln in einem kleinen Volumen dest. Wasser oder 0,5 N Salzsäure bzw. Natronlauge gelöst. Die Lösungen 2, 3 und 4 wurden mit Natronlauge auf pH=6,0 eingestellt und wie die Lösungen 1, 5 und 6 separat im Autoklaven während 15 Min. bei 120° C sterilisiert. Nach Mischen der Teillösungen, Einstellen des pH auf 6,8—6,9, Zusetzen der Wachststoffe und Verteilen des fertigen Substrats auf die trocken sterilisierten Kulturröhrchen, wurden diese im strömenden Dampf während 20 Min. erhitzt.

Zur Prüfung der Abhängigkeit des Wachstums von (+)-Biotin und *p*-Aminobenzoessäure wurde 0,001 µg des ersten bzw. 0,05 µg des letzten Wachstoffs pro ml der synthetischen Nährlösung I zugesetzt.

Zur Herstellung der Nährlösung I wurden analysenreine Salze der Firma Merck (Darmstadt) benutzt. Die Glucose, Aminosäuren und Wachststoffe bezogen wir von der Firma Hoffmann-La Roche (Basel).

Nährlösung II.

Dieses Substrat ist eine Modifikation der von Karnicki und Dorner (1934) zur Prüfung der Gärfähigkeit der Milchsäurebakterien empfohlenen Nährlösung. Es hat die folgende Zusammensetzung:

Fleischabsud	1000 ml
Pepton »Witte« für bakteriologische Zwecke	10,0 g
Pepton »Merck« für bakteriologische Zwecke	5,0 g
Hefeextrakt »Difco« für bakteriologische Zwecke	5,0 g
D(+)-Glucose	20,0 g

Der Fleischabsud wurde durch Kochen von 500 g fettfreiem zerkleinertem Rindfleisch mit 1000 ml dest. Wasser während 2 Stunden im Dampftopf, Filtrieren der Brühe und Ergänzung des verdampften Wassers auf 1000 ml erhalten. Nach Einstellen des pH auf 6,8—6,9 mit Natronlauge und Abfüllen wurde das fertige Substrat dreimal je 20 Min. im Dampftopf erhitzt.

Nähragar:

Dieses Substrat (Schrägagarröhrchen) diente zur Gewinnung des Impfmateri- als. Es enthielt 2 % Agar nebst den Bestandteilen der Nährlösung II.

Die zur Impfung verwendeten Sporensuspensionen wurden durch Aufschwemmung des Bakterienmaterials der Strichkulturen in sterilem dest. Wasser und Pasteurisation der Suspensionen bei 78° C während 10 Min. gewonnen. In zahlreichen Fällen wurde das Wachstum der *Clostridium*-Stämme mit und ohne Zusatz von Wachststoffen nach Impfung mit Sporensuspensionen, deren Konzentrationen sich wie 1 : 0,01 : 0,0001 verhielten, parallel untersucht. Dabei wurde die Ausgangssuspension pasteurisiert und mit dest. Wasser verdünnt.

Nach Impfung mit 0,05 ml Sporensuspension wurden die Kulturröhrchen nach Ritter und Dorner (1932) mit Pyrogallol (1 ml 20 % Lösung) und Soda (1 ml einer

Tabelle 1. pH-Werte der nicht geimpften Substrate nach Aufbewahrung während 5—15 Tage bei 37° C. Anfangs-pH=6,8—6,9.

Prüfung Nr.	Nährlösung I				Nährlösung II
	Kein Wuchs- stoffzusatz	(+)-Biotin 0,001 µg/ml	p-Amino- benzoesäure 0,05 µg/ml	(+)-Biotin 0,001 µg/ml p-Amino- benzoesäure 0,05 µg/ml	
3	6,64	6,62	6,64	6,66	6,43
4	6,45	6,56	6,43	6,55	6,12
5	6,43	6,51	6,35	6,36	6,08
6 }	6,44	6,37	—	—	6,19
		6,47			6,22
					6,15
					6,11
7 }	6,40	6,46	6,37	6,37	6,06
			6,37	6,39	
10	6,72	6,74	6,75	6,77	6,12
11	6,68	6,64	6,68	6,66	—
12 }	6,75	6,73	6,78	6,73	6,15
		6,72	6,75	6,74	
13 }	6,70	6,68	6,66	6,71	6,26
		6,78	6,78	6,82	
14	6,40	6,39	6,57	6,46	6,07
16 }	6,50	6,50	6,59	6,53	—
		6,53	6,70	6,64	
		6,64	6,67		
Mittel	6,56 s = 0,13	6,59 s = 0,11	6,61 s = 0,13	6,60 s = 0,13	6,16 s = 0,07

bei Zimmertemperatur gesättigten Lösung) anaerob verschlossen und bei 37° C bebrütet. Durch die starke Gasbildung wurden die als Abschluss dienenden Gummistopfen der gärenden Kulturen häufig explosiv aus den Röhrchen herausgetrieben.

Die Kulturen in Nährlösung II setzten wir an, um zu prüfen, ob die zur Impfung der synthetischen Substrate verwendeten Sporensuspensionen zur normalen Entwicklung in einem »natürlichen« Substrat fähig waren.

Ergebnisse und Diskussion

Nach Bebrütung während 5—15 Tage wurde das Wachstum der *Clostridium*-Stämme nach der Menge des Sediments und der Stärke der Trübung der Kulturen geschätzt und nach einer fünfteiligen Skala angegeben, wobei +++ sehr starkes, ++ mittelmässiges, + schwaches, ± fragliches und — kein Wachstum bezeichnete.

Ferner wurde der pH-Wert der Substrate direkt in den Kulturröhrchen

Tabelle 2. Die Gruppen von frisch isolierten *Clostridium*-Stämmen, welche sich auf Grund des Wachstums in den verschiedenen Substraten unterscheiden lassen. Bezeichnungen: +++ sehr starkes, ++ mittelmässiges, + schwaches, ± fragliches und — kein Wachstum.

Gruppe	Nährlösung I				Nährlösung II	Anzahl Stämme
	Kein Wuchsstoffzusatz	(+)-Biotin 0,001 µg/ml	p-Amino- benzoesäure 0,05 µg/ml	(+)-Biotin 0,001 µg/ml p-Amino- benzoesäure 0,05 µg/ml		
I	— oder ± oder +	+++	— oder ± oder +	+++	+++	105
II	+++	+++	+++	+++	+++	25
III	± oder +	++	± oder +	++	++ oder +++	48
IV	++	++	++	++	++	6
	+++	+++	+++	+++	+++	
V	± oder +	++	++	+++	+++	7
		+++	+++			

potentiometrisch mit Hilfe einer Glaselektrode ermittelt. Als Bezugselektrode diente eine gesättigte Kalomelelektrode und als Messgerät ein Polymetron-Potentiometer. Gleichzeitig mit den geimpften Kulturen wurden nicht geimpfte Substrate während 5—15 Tage bei 37° C aufbewahrt. Die pH-Werte dieser Substrate nach der Bebrütung sind in Tabelle 1 zusammengestellt. Es ist ersichtlich, dass pH durchgehends gesunken ist und zwar um 0,1—0,5 in Nährlösung I und um 0,4—0,8 in Nährlösung II.

In 16 zeitlich getrennten Prüfungsreihen wurden insgesamt 191 frisch isolierte *Clostridium*-Stämme untersucht. Dabei wurde ein und derselbe Stamm häufig mehrmals geprüft. Auf Grund des in den verschiedenen Substraten festgestellten Wachstums konnten dabei die in Tabelle 2 aufgeführten 5 Gruppen (I—V) von Stämmen unterschieden werden.

Gruppe I. Diese Gruppe umfasst diejenigen Stämme, welche in der synthetischen Nährlösung I in Anwesenheit des (+)-Biotins und der Kombination dieses Wuchsstoffs mit p-Aminobenzoesäure eine sehr starke, dagegen ohne Wuchsstoffzusatz und in Gegenwart von p-Aminobenzoesäure allein keine oder eine fragliche bis schwache Vermehrung zeigten. Zu dieser Gruppe gehörten nicht weniger als 105 Stämme, d.h. 55 % der geprüften frisch iso-

Tabelle 3. Die pH-Gruppen (1—5), in welche sich die *Clostridium*-Stämme der Gruppe I teilen lassen.

Gruppen 1 und 2: Ausgeprägte Buttersäurebakterien.

Gruppen 3 und 4: Intermediäre Formen.

Gruppe 5: Typische Butylbakterien.

Gruppe Nr.	Substrat					Anzahl Stämme	
	Nährlösung I				Nähr- lösung II		
	Kein Wuchs- stoffzusatz	(+)-Biotin 0,001 µg/ml	p-Amino- benzoesäure 0,05 µg/ml	(+)-Biotin 0,001 µg/ml p-Amino- benzoesäure 0,05 µg/ml			
I: 1	Max.: Mittel: ... δ: Min.:	6,74 6,33 0,17 6,02	4,98 4,84 0,08 4,68	6,56 6,25 0,17 6,00	5,02 4,82 0,12 4,50	4,82 4,67 0,08 4,52	27
I: 2	Max.: Mittel: ... δ: Min.:	5,93 5,44 0,22 5,00	4,98 4,82 0,09 4,63	5,94 5,49 0,24 5,01	4,97 4,81 0,09 4,64	4,78 4,62 0,05 4,56	24
I: 3	Max.: Mittel: ... δ: Min.:	6,52 6,23 0,14 6,00	5,86 5,38 0,15 5,07	6,63 6,21 0,16 5,98	5,86 5,36 0,17 5,05	5,78 5,08 0,23 4,70	30
I: 4	Max.: Mittel: ... δ: Min.:	5,98 5,78 0,10 5,44	5,49 5,24 0,09 5,06	5,98 5,72 0,14 5,46	5,42 5,21 0,11 5,08	5,26 4,90 0,16 4,53	13
I: 5	Max.: Mittel: ... δ: Min.:	6,46 6,18 0,15 6,00	6,50 6,19 0,12 5,98	6,38 6,18 0,10 6,03	6,46 6,20 0,13 6,00	6,15 5,85 0,20 5,63	11

lierten Reinkulturen (vgl. Tabelle 3). Zur Gruppe I konnten ferner 4 als Bezugsorganismen dienende Laboratorium-Stämme und zwar *Cl. acetobutylicum* McCoy, Fred, Peterson et Hastings (Nr. 9594 von der »Amer. Type Culture Collection«), *Cl. beijerinckii* Donker (von Delft), *Cl. butylicum* (Beijerinck) Donker (von Delft) und *Cl. pasteurianum* Winogradsky (von Delft) gezählt werden.

Gruppe II. Zu dieser Gruppe zählten wir die Stämme, welche in den mit (+)-Biotin oder einem Gemisch von (+)-Biotin und p-Aminobenzoesäure versetzten Kulturen auf synthetischem Substrat ein sehr starkes, in Abwesenheit dieser Wachstoffsowie in Anwesenheit der p-Aminobenzoesäure allein aber nur ein mittelmässiges Wachstum zeigten. So verhielten sich insgesamt 25 Stämme, d.h. 13 % aller geprüften Reinkulturen (vgl. Tabelle 4).

Tabelle 4. Die pH-Gruppen (2—4), welche innerhalb der Gruppe II der *Clostridium*-Stämme unterschieden werden können.

Gruppe 2: Ausgeprägte Buttersäurebakterien.

Gruppen 3 und 4: Intermediäre Formen.

Gruppe Nr.	Substrat					Anzahl Stämme	
	Nährlösung I				Nähr- lösung II		
	Kein Wuchs- stoffzusatz	(+)-Biotin 0,001 µg/ml	p-Amino- benzoesäure 0,05 µg/ml	(+)-Biotin 0,001 µg/ml p-Amino- benzoesäure 0,05 µg/ml			
II: 2	Max.:..... Mittel: ... s: Min.:	5,04 4,88 0,09 4,73	4,91 4,74 0,05 4,56	4,95 4,84 0,08 4,71	4,93 4,75 0,04 4,69	4,67 4,44 0,13 4,27	13
II: 3	Max.:..... Mittel: ... s: Min.:	6,32 6,22 0,09 6,08	5,76 5,51 0,22 5,20	6,55 6,30 0,16 6,06	5,90 5,56 0,22 5,22	5,12 4,96 0,11 4,87	4
II: 4	Max.:..... Mittel: ... s: Min.:	5,88 5,64 0,11 5,45	5,42 5,27 0,10 5,10	5,88 5,73 0,14 5,51	5,51 5,23 0,11 5,02	5,12 4,93 0,13 4,62	8

Gruppe III. Die Stämme dieser Gruppe wiesen in der synthetischen Nährlösung in Gegenwart des (+)-Biotins bzw. der Kombination von (+)-Biotin und p-Aminobenzoesäure ein mittelmässiges, in den Kulturen, welche wuchsstofffrei waren oder nur p-Aminobenzoesäure enthielten, ein fragliches bis schwaches Wachstum auf. Zu dieser Gruppe konnten 48, d.h. 25 % der untersuchten, frisch isolierten *Clostridium*-Stämme gezählt werden (vgl. Tabelle 5).

Die Gruppen I—III umfassen somit die *Clostridium*-Stämme, bei welchen ein wachstumsfördernder Einfluss des (+)-Biotins einwandfrei festgestellt werden konnte. Die p-Aminobenzoesäure allein übte auf diese Stämme keine Wuchsstoffwirkung aus. Sie vermochte auch nicht die Stimulierung der Vermehrung der Bakterienzellen durch (+)-Biotin augenfällig zu steigern. Die Zahl der so charakterisierten Stämme betrug 178 und machte mithin 93 % der geprüften frisch isolierten Reinkulturen aus.

Gruppe IV. Die Bakterienstämme dieser Gruppe zeigten in der synthetischen Nährlösung I das gleiche Wachstum unabhängig davon, ob die Kulturen vor der Impfung wuchsstofffrei waren oder (+)-Biotin und p-Aminobenzoesäure, einzeln oder gleichzeitig zugesetzt, enthielten. Die Vermehrung der Bakterienzellen war durchgehends in den synthetischen Substraten

Tabelle 5. Die pH-Gruppen (1—3 und 5), in welche die *Clostridium*-Stämme der Gruppe III eingeteilt werden können.

Gruppen 1 und 2: Ausgeprägte Buttersäurebakterien.

Gruppe 3: Intermediäre Formen.

Gruppe 5: Typische Butylbakterien.

Gruppe Nr.	Substrat					Anzahl Stämme	
	Nährlösung I				Nähr- lösung II		
	Kein Wuchs- stoffzusatz	(+)-Biotin 0,001 µg/ml	p-Amino- benzoesäure 0,05 µg/ml	(+)-Biotin 0,001 µg/ml p-Amino- benzoesäure 0,05 µg/ml			
III: 1	Max.: ...	6,57	4,98	6,33	4,96	4,74	5
	Mittel:...	6,28	4,93	6,13	4,68	4,70	
	Ø:	0,11	0,08	0,11	0,10	0,04	
	Min.: ...	6,10	4,84	6,07	4,55	4,66	
III: 2	Max.: ...	5,94	4,94	5,98	4,95	4,69	13
	Mittel:...	5,49	4,83	5,54	4,82	4,48	
	Ø:	0,21	0,07	0,27	0,11	0,16	
	Min.: ...	5,10	4,69	5,11	4,56	4,28	
III: 3	Max.: ...	6,64	5,94	6,66	5,84	5,87	19
	Mittel:...	6,29	5,42	6,12	5,40	5,12	
	Ø:	0,16	0,20	0,11	0,24	0,25	
	Min.: ...	6,02	5,00	6,00	5,00	4,66	
III: 5	Max.: ...	6,73	6,65	6,78	6,80	5,95	11
	Mittel:...	6,29	6,16	6,34	6,19	5,70	
	Ø:	0,18	0,16	0,18	0,16	0,15	
	Min.: ...	6,04	5,99	6,00	5,96	5,27	

gleich wie in der »natürlichen« Nährlösung II. So verhielten sich 6 Stämme, d.h. nur 3 % der untersuchten Reinkulturen. Ob es sich in diesen Fällen um Bakterientypen handelt, welche das Aminosäuregemisch der synthetischen Nährlösung ohne irgend welchen Zusatz von Wuchsstoffen assimilieren können, oder um Stämme, die auf dem erwähnten Gemisch einen so geringen Wuchsstoffbedarf haben, dass er aus dem Impfmateriel gedeckt werden kann, bleibt vorläufig eine offene Frage. Immerhin deuten unsere Versuche nicht darauf hin, dass es sich um eine Auxo-Autotrophie »degenerierter« Organismen im Sinne Perlman's (1948) handelt (vgl. Tabelle 6).

Gruppe V. Zu dieser wurden die Stämme gezählt, welche ohne Zusatz von Wuchsstoffen nur ein fragliches bis schwaches Wachstum zeigten, in Anwesenheit des (+)-Biotins, der p-Aminobenzoesäure oder einer Kombination der beiden Vitamine dagegen eine mittelmässige bis sehr starke Vermehrung aufwiesen. Die Zahl der Stämme, auf welche p-Aminobenzoesäure im syn-

Tabelle 6. Die pH-Gruppen (2 und 4), welche sich innerhalb der Gruppe IV der *Clostridium*-Stämme unterscheiden lassen.

Gruppe 2: Ausgeprägte Buttersäurebakterien.

Gruppe 4: Intermediäre Formen.

Gruppe Nr.	Substrat					Anzahl Stämme
	Nährlösung I				Nähr- lösung II	
	Kein Wuchs- stoffzusatz	(+)-Biotin 0,001 µg/ml	p-Amino- benzoesäure 0,05 µg/ml	(+)-Biotin 0,001 µg/ml p-Amino- benzoesäure 0,05 µg/ml		
IV: 2	Max.: ...	5,03	4,84	5,07	4,85	3
	Mittel:...	4,96	4,81	4,96	4,80	
	̄:	0,05	0,03	0,09	0,04	
	Min.: ...	4,90	4,76	4,95	4,76	
IV: 4	Max.: ...	5,77	5,74	5,74	5,54	3
	Mittel:...	5,64	5,34	5,37	5,26	
	̄:	0,19	0,17	0,16	0,17	
	Min.: ...	5,44	5,14	5,17	5,10	

Tabelle 7. Die pH-Gruppen (2 und 4), in welche die Gruppe V der *Clostridium*-Stämme geteilt werden kann.

Gruppe 2: Ausgeprägte Buttersäurebakterien.

Gruppe 4: Intermediäre Formen.

Gruppe Nr.	Substrat					Anzahl Stämme
	Nährlösung I				Nähr- lösung II	
	Kein Wuchs- stoffzusatz	(+)-Biotin 0,001 µg/ml	p-Amino- benzoesäure 0,05 µg/ml	(+)-Biotin 0,001 µg/ml p-Amino- benzoesäure 0,05 µg/ml		
V: 2	Max.:	5,94	5,01	5,07	5,06	3
	Mittel: ...	5,47	4,84	4,85	4,79	
	Ø:	0,33	0,11	0,11	0,11	
	Min.:	5,03	4,64	4,62	4,56	
V: 4	Max.:	5,98	5,58	5,77	5,46	4
	Mittel: ...	5,77	5,37	5,36	5,27	
	Ø:	0,13	0,10	0,17	0,08	
	Min.:	5,51	5,17	5,02	5,03	

thetischen Substrat somit eine Wuchsstoffwirkung ausübt, betrug 7, d.h. nur 3—4 % der Gesamtzahl der geprüften frisch isolierten *Clostridium*-Kulturen (vgl. Tabelle 7).

Aus den erwähnten Ergebnissen geht eindeutig die grosse Bedeutung des (+)-Biotins für die Vermehrung der aus den verschiedensten Materialien frisch isolierten *Clostridium*-Stämme hervor. Insofern nicht bereits bei der Reinzüchtung der Bakterien eine Auswahl (»selection») oder Mutationen (»genic mutations») stattfinden, wodurch die Zusammensetzung der Populationen stark geändert wird, sollten die natürlichen Populationen der Buttersäure- und Butylbakterien mithin grösstenteils (zu 90—95 %) aus (+)-Biotin-heterotrophen Zellen bestehen. Dabei tritt die Biotin-Heterotrophie sogar in Substraten auf, welche die meisten Aminosäuren des Eiweisses der lebenden Zellen enthalten (Nährlösung I: 20 Aminosäuren). Auf Grund der Mannigfaltigkeit der zur Anreicherung bzw. Isolierung der Stämme verwendeten Substrate (Richard 1948, 1950; vgl. oben), darf angenommen werden, dass die Zusammensetzung der geprüften *Clostridium*-Populationen bezüglich auxo-autotropher bzw. auxo-heterotropher Typen derjenigen der natürlichen Populationen weitgehend entspricht. Der Prozentsatz an Stämmen von auxo-autotrophem Charakter bzw. mit *p*-Aminobenzoesäure-Heterotrophie war sehr gering (3 bzw. 3—4 %). Bei den letzten Typen konnte *p*-Aminobenzoesäure durch (+)-Biotin ersetzt werden. Bemerkenswert ist, dass der geprüfte Laboratorium-Stamm von *Cl. acetobutylicum* (Nr. 9594 von der »Amer. Type Culture Collection») nach Zusatz von (+)-Biotin allein in Nährlösung I zur normalen Vermehrung gebracht werden konnte. Wie bereits erwähnt, wurde mehrmals festgestellt, dass Stämme dieser Art eine synthetische Nährlösung, welche Ammonsulfat, Ammonsulfat+Ammonphosphat, Asparagin oder Asparagin+Ammonsulfat als Stickstoffquelle enthält, erst nach gleichzeitigem Zusatz von Biotin und *p*-Aminobenzoesäure assimilieren können. Die Annahme liegt nahe, dass der letzte Wuchsstoff durch eine oder mehrere der in Nährlösung I vorhandenen Aminosäuren ersetzt werden kann. Dies wäre im Hinblick auf die von Lampen und Peterson (1944) ausgearbeitete Methode zur quantitativen Bestimmung des Gehaltes natürlicher Materialien an *p*-Aminobenzoesäure mit Hilfe von *Cl. acetobutylicum* von gewissem Interesse.

Auf Grund der pH-Werte, welche nach beendeter Gärung für die synthetischen Substrate festgestellt wurden, konnten 5 Gruppen von Kulturen (1—5) unterschieden werden (vgl. Tabellen 3—7).

Gruppe 1. Diese Gruppe ist dadurch charakterisiert, dass der pH-Wert der Kulturen mit mittelmässigem bis sehr starkem Bakterienwachstum auf durchschnittlich 4,68—4,93 ($\delta=0,08$ —0,12; Max.=4,96—5,02; Min.=4,50—4,84) gesunken war, während das pH der Kulturen mit keinem oder fraglichem bis schwachem Wachstum im Mittel 6,13—6,33 ($\delta=0,11$ —0,17; Max.=6,33—6,74; Min.=6,00—6,10) betrug (Tabellen 3 und 5).

Gruppe 2. Die Kulturen dieser Gruppe, welche durch mittelmässiges bis sehr starkes Wachstum gekennzeichnet waren, hatten einen pH-Wert von durchschnittlich 4,74—4,96 ($\delta=0,03—0,11$; Max.=4,84—5,07; Min.=4,56—4,95). Für die Kulturen mit keiner oder fraglicher bis schwacher Bakterienvermehrung wurden pH-Werte von durchschnittlich 5,44—5,54 ($\delta=0,21—0,33$; Max.=5,93—5,98; Min.=5,00—5,11) ermittelt (Tabellen 3—7).

Die Gruppen 1 und 2 umfassen somit *Clostridium*-Formen, welche durch kräftige Säurebildung charakterisiert sind, wobei diese bei den Stämmen der ersten Gruppe eindeutig an eine starke Zellvermehrung geknüpft ist, während sich bei den Formen der letzten Gruppe sogar in den Kulturen mit keiner oder nur sehr bescheidener Vermehrung eine beträchtliche Säuerung feststellen lässt. Wenn zur Impfung der synthetischen Substrate Sporensuspensionen zur Anwendung kamen, welche auf das hundert- oder zehntausendfache verdünnt waren, fand in den wuchsstofffreien bzw. mit *p*-Aminobenzoesäure allein versetzten synthetischen Substraten auch durch die Stämme der Gruppe 2 praktisch keine Säurebildung statt, während in den wuchsstoffhaltigen (Biotin bzw. Biotin + *p*-Aminobenzoesäure) Nährlösungen, wo kräftiges Bakterienwachstum einsetzte, die Säuerung normal verlief und somit sehr stark war. Einige Ergebnisse der Versuche über die Abhängigkeit der pH-Verschiebung von der Konzentration der Impfsuspensionen (1:0,01:0,0001) sind in Tabelle 8 angeführt. Die Stämme 150, 194, 196 und 208 sind Vertreter der zur kräftigen Säureproduktion fähigen Formen.

Die *Clostridium*-Formen der Gruppen 1 und 2 sind typische Buttersäure-Essigsäure-Bildner, welche nebst den Fettsäuren nur sehr geringe Mengen neutrale Produkte erzeugen. Dies geht aus den in Tabellen 9 und 10 angeführten Daten für die Vergärung der Glucose in synthetischem bzw. hefeextrakthaltigem Substrat durch Stamm 208 hervor (vgl. Wikén 1951; Wikén, Richard und Aebi 1952).

Gruppe 3. In den Kulturen dieser Gruppe auf synthetischem Substrat, welche mit (+)-Biotin oder der Kombination von (+)-Biotin und *p*-Aminobenzoesäure versetzt waren und mittelmässiges bis sehr starkes Wachstum zeigten, sank pH auf durchschnittlich 5,36—5,56 ($\delta=0,15—0,24$; Max.=5,76—5,94; Min.=5,00—5,22). Für die Kulturen ohne Wuchsstoffzusatz oder mit *p*-Aminobenzoesäure allein, welche kein oder fragliches bis mittelmässiges Wachstum aufwiesen, wurde ein pH-Wert von durchschnittlich 6,12—6,30 ($\delta=0,09—0,16$; Max.=6,32—6,66; Min.=5,98—6,08) ermittelt (Tabellen 3—5).

Gruppe 4. In dieser Gruppe zeigten die mittelmässig bis sehr stark gewachsenen Kulturen auf synthetischem Substrat mit Zusatz von (+)-Biotin oder (+)-Biotin plus *p*-Aminobenzoesäure einen pH-Wert von durchschnitt-

Tabelle 8. Die Abhängigkeit des pH-Wertes der bebrüteten Substrate von der Konzentration (1:0,01:0,0001) der zur Impfung der Kulturen verwendeten Sporensuspensionen. Die Stämme 150, 194, 196 und 208 sind ausgeprägte Buttersäurebakterien, welche praktisch nur Buttersäure und Essigsäure erzeugen, während die Stämme 98 und 157 intermediäre Formen sind, die nebst Fettsäuren beträchtliche Mengen neutrale Produkte (*n*-Butylalkohol und Isopropylalkohol oder Aceton) bilden. Sämtliche Stämme gehören zur Gruppe I.

Stamm Nr.	Nährlösung I				Nährlösung II	
	Kein Wuchs- stoffzusatz	(+)-Biotin 0,001 µg/ml	<i>p</i> -Amino- benzoesäure 0,05 µg/ml	(+)-Biotin 0,001 µg/ml <i>p</i> -Amino- benzoesäure 0,05 µg/ml		
98	{ 1 0,01 0,0001	5,62 6,42 6,32	5,16 5,18 5,30	5,48 5,72 6,20	5,20 5,28 5,21	5,26 5,25 5,25
150	{ 1 0,01 0,0001	5,37 6,18 6,57	4,68 4,60 4,62	5,51 5,84 6,26	4,64 4,50 4,60	4,78 — —
157	{ 1 0,01 0,0001	5,66 5,92 6,18	5,38 5,14 5,44	5,83 5,80 6,18	5,09 5,12 5,16	— — —
194	{ 1 0,01 0,0001	5,56 6,30 6,29	4,80 5,20 4,88	5,50 6,20 6,31	4,81 5,18 4,68	4,65 5,16 4,64
196	{ 1 0,01	5,56 6,06	4,80 4,71	5,64 6,20	4,79 4,73	4,68 4,72
208	{ 1 0,01 0,0001	5,93 6,20 6,35	4,64 4,65 4,68	5,62 5,98 5,98	4,67 4,61 4,54	— — —

lich 5,21—5,37 ($\delta=0,09-0,17$; Max.=5,42—5,74; Min.=5,02—5,17). Die Kulturen ohne Wuchsstoffzusatz mit keiner oder fraglicher bis mittelmässiger Zellvermehrung hatten im Mittel einen pH-Wert von 5,64—5,78 ($\delta=0,10-0,19$; Max.=5,77—5,98; Min.=5,44—5,51), während die entsprechenden Kulturen mit *p*-Aminobenzoesäure ein pH von durchschnittlich 5,36—5,73 ($\delta=0,14-0,17$; Max.=5,74—5,98; Min.=5,02—5,51) aufwiesen (Tabellen 3—4 und 6—7).

Die Stämme der Gruppen 3 und 4 sind somit durch eine mittelmässige Säurebildung gekennzeichnet. In jener Gruppe zeigen nur die Kulturen mit kräftiger Vermehrung Säuerung, während in dieser Gruppe eine gewisse Säurebildung feststellbar ist, obwohl in den betreffenden Kulturen keine oder nur eine sehr bescheidene Vermehrung stattgefunden hat. Aus den Daten, welche in Tabelle 8 für die Stämme 98 und 157 angeführt sind, geht hervor, dass die Säurebildung in nicht oder nur schlecht gewachsenen Kul-

Tabelle 9. Vergärung der Glucose in synthetischer Nährlösung durch *Clostridium*-Formen. Stamm 208 ist eine ausgeprägte Buttersäurebakterie (frisch isoliert), Stamm 174 eine intermediäre Form (frisch isoliert) und *Cl. butylicum* eine typische Butylbakterie (vgl. Wikén 1951; Wikén, Richard und Aebi 1952).

Gärprodukte in mM pro 100 mM vergorene Glucose in Nährlösung I (mit (+)-Biotin)						
Produkte	Stamm 208 pH-Gruppen 1 und 2		Stamm 174 pH-Gruppen 3 und 4		<i>Clostridium butylicum</i> pH-Gruppe 5	
	Mittel ¹	ø	Mittel ¹	ø	Mittel ²	ø
<i>n</i> -Buttersäure	91,1	2,6	50,6	2,5	8,0	0,3
Essigsäure	36,0	3,6	53,3	1,4	29,2	0,0
«Ameisensäure» ...	2,2	0,2	4,0	0,3	2,8	0,1
<i>n</i> -Butylalkohol	0,0	0,0	37,6	1,0	56,8	0,9
Aethylalkohol	3,3	0,2	1,4	0,2	1,3	0,6
Isopropylalkohol ...	0,0	0,0	4,1	0,5	24,6	2,6
Aceton	0,1	0,0	0,5	0,1	0,7	0,0
pH-Aenderung	6,88 ↓ 4,74—4,75		6,88 ↓ 5,12—5,16		6,95 ↓ 6,54—6,60	
Glucoseverbrauch %	96,0—96,1		81,0—86,2		96,9—97,5	

¹ Durchschnitt aus 3 parallelen Gärungen.

² Durchschnitt aus 2 parallelen Gärungen.

Tabelle 10. Vergärung der Glucose in Hefeextrakt durch *Clostridium*-Formen. Stamm 208 ist eine ausgeprägte Buttersäurebakterie (frisch isoliert), Stamm 174 eine intermediäre Form (frisch isoliert) und *Cl. butylicum* eine typische Butylbakterie (vgl. Wikén 1951; Wikén, Richard und Aebi 1952).

Gärprodukte in mM pro 100 mM vergorene Glucose in Hefeextrakt (Stämme 208 und 174) bzw. Hefeextrakt + 0,695 % K ₂ HPO ₄ (<i>Cl. butylicum</i>)						
Produkte	Stamm 208 pH-Gruppen 1 und 2		Stamm 174 pH-Gruppen 3 und 4		<i>Clostridium butylicum</i> pH-Gruppe 5	
	Mittel ¹	ø	Mittel ¹	ø	Mittel ¹	ø
<i>n</i> -Buttersäure	75,1	1,1	39,6	0,1	8,1	3,5
Essigsäure	22,2	3,5	29,7	0,0	24,7	1,8
»Ameisensäure« ...	1,2	0,4	6,0	0,1	—	—
<i>n</i> -Butylalkohol	0,0	0,0	61,6	1,1	63,1	1,0
Aethylalkohol	4,4	0,1	0,0	0,0	1,4	0,0
Isopropylalkohol ...	0,0	0,0	0,8	0,2	10,2	4,4
Aceton	0,0	0,0	0,0	0,0	0,7	0,0
pH-Aenderung	6,78 ↓ 4,15—4,17		6,78 ↓ 4,58—4,60		7,66 ↓ 5,74—6,08	
Glucoseverbrauch %	73,5—75,7		38,8—40,5		86,6—93,5	

¹ Durchschnitt aus 3 parallelen Gärungen.

turen in der Gruppe 4 gleich wie in der Gruppe 2 von der Konzentration der Impfsuspension abhängig ist.

Die Stämme der Gruppen 3 und 4 sind Butylbakterien, welche nebst Buttersäure und Essigsäure beträchtliche Mengen neutrale Produkte, v.a. *n*-Butylalkohol und Isopropylalkohol oder Aceton, bilden. Die Verteilung der sauren und neutralen Gärprodukte des frisch isolierten Stammes 174 auf Glucose in (+)-Biotin-haltiger synthetischer Nährlösung bzw. einem Hefeextraktsubstrat geht aus Tabellen 9 und 10 hervor (vgl. Wikén 1951; Wikén, Richard und Aebi 1952).

Gruppe 5. In dieser Gruppe hatten die mittelmässig bis sehr stark gewachsenen Kulturen auf synthetischem Substrat im Mittel einen pH-Wert von 6,16—6,20 ($\delta=0,12-0,16$; Max.=6,46—6,80; Min.=5,96—6,00) und diejenigen, welche keines oder fragliches bis schwaches Wachstum aufwiesen, einen Wert von durchschnittlich 6,18—6,34 ($\delta=0,10-0,18$; Max.=6,38—6,78; Min.=6,00—6,04). Vgl. Tabellen 3 und 5.

Die Gruppe 5 umfasst mithin Butylbakterien, welche nur geringe Mengen Fettsäuren, dagegen sehr grosse Quantitäten *n*-Butylalkohol und Isopropanol oder Aceton erzeugen. Als Beispiel dieser Organismen ist in Tabellen 9 und 10 *Clostridium butylicum* angeführt (vgl. Wikén 1951; Wikén, Richard und Aebi 1952).

Wie bereits erwähnt, beziehen sich die pH-Gruppen auf die in synthetischen Substraten gewachsenen Kulturen. In der »natürlichen« Nährlösung II konnten durchgehends tiefere pH-Werte festgestellt werden, wobei aber die Reihenfolge der Stämme die gleiche blieb. Die Ergebnisse der pH-Messungen in den Kulturen auf Nährlösung II lassen sich folgendermassen zusammenfassen (vgl. Tabellen 3—7):

Gruppe 1: Mittel=4,67—4,70 ($\delta=0,04-0,08$; Max.=4,74—4,82; Min.=4,52—4,66).

Gruppe 2: Mittel=4,44—4,62 ($\delta=0,05-0,16$; Max.=4,67—4,78; Min.=4,28—4,56).

Gruppe 3: Mittel=4,96—5,12 ($\delta=0,11-0,25$; Max.=5,12—5,87; Min.=4,66—4,87).

Gruppe 4: Mittel=4,90—5,10 ($\delta=0,02-0,16$; Max.=5,12—5,26; Min.=4,53—5,07).

Gruppe 5: Mittel=5,70—5,85 ($\delta=0,15-0,20$; Max.=5,95—6,15; Min.=5,27—5,63).

Das von uns auf den Bedarf an (+)-Biotin und *p*-Aminobenzoesäure geprüfte frisch isolierte *Clostridium*-Material umfasst somit einerseits ausgeprägte Buttersäurebakterien, welche praktisch ausschliesslich Buttersäure und Essigsäure bilden (Gruppen 1 und 2), anderseits typische Butylbakte-

Tabelle 11. Die Verteilung der 191 frisch isolierten *Clostridium*-Stämme auf die pH-Gruppen 1—5 und die Gruppen I—V, welche auf Grund des Wachstums in den geprüften Substraten unterschieden werden können.

Wuchsstoff- bedarf pH Gärungstypus	Gruppen I, II und III (+)-Biotin- heterotrophe Stämme	Gruppe IV Auxo-autotrophe oder durch einen sehr geringen Wuchs- stoffbedarf gekennzeichnete Stämme	Gruppe V (+)-Biotin- oder p-Aminobenzoessäure- heterotrophe Stämme
Gruppen 1 und 2 Ausgeprägte Buttersäure- bakterien	82	3	3
Gruppen 3 und 4 Intermediäre Formen	74	3	4
Gruppe 5 Ausgeprägte Butylbakterien	22	0	0

rien, welche sehr grosse Mengen *n*-Butylalkohol und Isopropylalkohol oder Aceton erzeugen (Gruppe 5), sowie intermediäre Formen (Gruppen 3 und 4). Die Verteilung der Typen ist wie folgt:

Gruppen 1 und 2: 88 Stämme oder 46 %

Gruppe 5: 22 Stämme oder 11,5 %

Gruppen 3 und 4: 81 Stämme oder 42,5 %

In Tabelle 11 sind die Ergebnisse der Wachstumsschätzungen mit denjenigen der pH-Messungen und Gärsubstratanalysen kombiniert. Es geht eindeutig hervor, dass nicht nur die ausgeprägten Butylbakterien, sondern auch die typischen Buttersäurebakterien und die intermediären Formen, welche in natürlichen Populationen vorkommen, weitgehend durch (+)-Biotin-Heterotrophie gekennzeichnet sind.

Summary

The present paper reports the results obtained in examining 191 freshly isolated saccharolytic *Clostridium* strains for growth under anaerobic conditions in a synthetic nutrient solution with or without addition of (+)-biotin and *p*-aminobenzoic acid. Further, the pH of the substrates was determined electrometrically after completed fermentation.

The strains were isolated from soil, sewage, green plants, silage, potato-, corn-, rye- and chestnut-mash, spoiled Emmentaler and process cheese,

faeces of cow and sheep, etc. The pure cultures were obtained from the pasteurized material *directly* by means of smear-cultures on glucose-meat infusion-agar, glucose-yeast water-agar, etc. or *after enrichment* on skim milk, glucose-skim milk, corn- and pea-broth, glucose-meat-peptone-broth, Winogradsky's solution, etc.

The pH measurements and analyses of the fermented substrates showed that out of the 191 strains thus isolated 88 (46 per cent) were true butyric acid bacteria producing great amounts of butyric and acetic acids, along with traces of neutral compounds, whereas 22 strains (11.5 per cent) were butanol-isopropanol or butanol-acetone bacteria capable of forming large quantities of neutral products, together with only small amounts of fatty acids. 81 strains (42.5 per cent) were found to be intermediate types.

The synthetic medium used contained 20 amino acids in addition to glucose, ammonium acetate and mineral salts. (+)-biotin and *p*-aminobenzoic acid were supplied in amounts of 0.001 μ g and 0.05 μ g, respectively, per ml of substrate. In order to check the quality of the pasteurized spore suspensions used for inoculation parallel cultures were grown in a modified Karnicki-Dorner medium.

It was demonstrated conclusively that 178 strains (93 per cent) were heterotrophic for (+)-biotin, whereas 6 strains (3 per cent) showed auxo-autotrophic tendencies, and 7 strains (3—4 per cent) required (+)-biotin or *p*-aminobenzoic acid for normal growth in the synthetic substrate mentioned.

The end products of fermentation found in the synthetic medium supplied with (+)-biotin are, qualitatively and quantitatively, the same as those observed in parallel cultures on a glucose-yeast extract substrate.

As far as selection and genic mutations involving changes from an autotrophic to a heterotrophic growth-factor habit do not occur in the media employed for isolating the strains and growing the subcultures used in preparing the spore suspensions, we may conclude that the natural populations of butyric acid as well as butanol-isopropanol and butanol-acetone bacteria of the genus *Clostridium* contain a very great number of strains requiring an exogenous supply of (+)-biotin for normal growth even in media containing most of the amino acids obtained on hydrolysis of bacterial proteins. The number of strains with auxo-autotrophic tendencies is obviously very small. In some strains a replacement of (+)-biotin by *p*-aminobenzoic acid seems possible.

Diese Arbeit ist ein Teil der Untersuchungen über die Physiologie der Buttersäure- und Butylbakterien, welche aus den Arbeitsbeschaffungskrediten des Bundes zur Förderung der wissenschaftlichen Forschung unterstützt werden. Den zuständigen Behörden sind wir zu grossem Dank verpflichtet.

Herrn Prof. Dr. A. J. Kluyver, Delft, stellte uns je einen Stamm von *Cl. beijerinckii* Donker, *Cl. butylicum* (Beijerinck) Donker und *Cl. pasteurianum* Winogradsky zur Verfügung. Wir sprechen Herrn Prof. Kluyver unseren herzlichen Dank aus.

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Literatur

- Bergey's Manual of determinative bacteriology (Herausgegeben von Breed, R. S., Murray, E. G. D. & Hitchens, A. P.). — Baltimore 1948.
- Block, R. J. & Bolling, D.: The amino acid composition of proteins and foods. — Springfield, Ill. 1947.
- Karnicki, F. & Dorner, W.: Die Milchsäurebakterienflora der in der schweizerischen Käserei üblichsten Lab- und Kulturarten. — Landw. Jahrbuch der Schweiz. 48. Jahrg.: 67. 1934.
- Lampen, J. O. & Peterson, W. H.: Biotin and para-aminobenzoic acid as growth factors for the acetone-butanol organism, *Clostridium acetobutylicum*. — Journ. Amer. Chem. Soc. 63: 2283. 1941.
- — Growth factor requirements of Clostridia. — Arch. Biochem. 2: 443. 1943.
- — The determination of p-aminobenzoic acid by assay with *Clostridium acetobutylicum*. — Journ. Biol. Chem. 153: 193. 1944.
- Möller, E. F.: Das Wuchsstoffsystem der Milchsäurebakterien. — Zeitschr. physiol. Chem. 260: 246. 1939.
- Perlman, D.: Desthiobiotin and O-heterobiotin as growth factors for »normal» and »degenerate» strains of Clostridia. — Arch. Biochem. 16: 79. 1948.
- Peterson, W. H., McDaniel, L. E. & McCoy, E.: Biotin requirements of Clostridia and assay of biological materials for biotin. — Journ. Biol. Chem. 133. Sci. Proceed. 34: 75. 1940.
- Reyes-Teodoro, R. & Mickelson, M. N.: Growth factor requirements of saccharolytic butyl alcohol-acetone bacteria. — Arch. Biochem. 6: 471. 1945.
- Richard, O.: Variation in morphological and biochemical characteristics of anaerobic butyric acid bacteria. — Nature 162: 463. 1948.
- Zur Identifizierung der beweglichen Buttersäurebakterien. — Schweiz. Zeitschr. allgem. Pathol. Bakteriologie. 13: 560. 1950.
- Ritter, W. & Dorner, W.: Behebung eines wichtigen Nachteils des anaeroben Pyrogallolverchlusses. — Zentralbl. Bakt. I. Abt. 125: 379. 1932.
- Rubbo, S. D. & Gillespie, J. M.: Para-amino benzoic acid as a bacterial growth factor. — Nature 146: 838. 1940.
- Snell, E. E. & Williams, R. J.: Biotin as a growth factor for the butyl alcohol producing anaerobes. — Journ. Amer. Chem. Soc. 61: 3594. 1939.
- Wikén, T.: Ueber aktuelle Fragen der landwirtschaftlichen Bakteriologie und Gärungsbiologie. — Schweiz. Landw. Monatshefte. 29. Jahrg.: 118. 1951.
- Wikén, T., Richard, O. & Aebi, H.: Ueber die Vergärung der Glucose in einem synthetischen Substrat durch *Clostridium*-Formen. — Schweiz. Zeitschr. allgem. Pathol. Bakteriologie. 15. 1952 (im Druck).
- Winogradsky, S.: *Clostridium Pastorianum*, seine Morphologie und seine Eigenschaften als Buttersäureferment. — Zentralbl. Bakt. II. Abt. 9: 43. 1902.

Remarks on the Submicroscopical Structure of Eggs and Spermatozoids of *Fucus* and Related Genera

By

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The formation of eggs and spermatozoids as well as the fertilization of *Fucus* and related genera has been studied by several scientists. Classical and well known are the researches of Thuret (14), whose excellent pictures are copied in several textbooks. Among other investigations that of Farmer and Williams (4) may also be mentioned. These authors showed the copulation of the male and female nuclei of *Halidrys siliquosa*. The germination of the fertilized eggs as well as the origin of the polarity has been described by Kniep (5). On this latter subject extensive researches have been carried out recently, especially by Whitaker (15).

None of these investigators gave any details about the submicroscopical structure of the gametes. This writer has tried to describe the structure of the surface layers of the eggs and the mechanism of cell-wall formation after fertilization (cf. Levring 6, 7). The present paper will give a summary of these investigations, which have been continued since the two first papers were published.

Unfertilized eggs

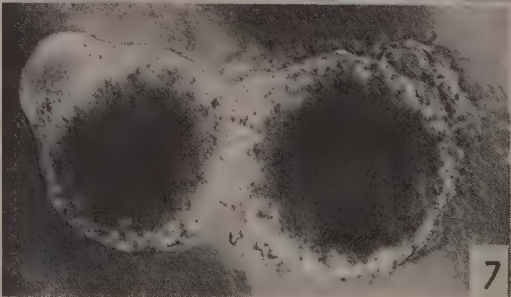
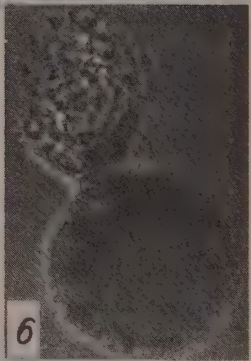
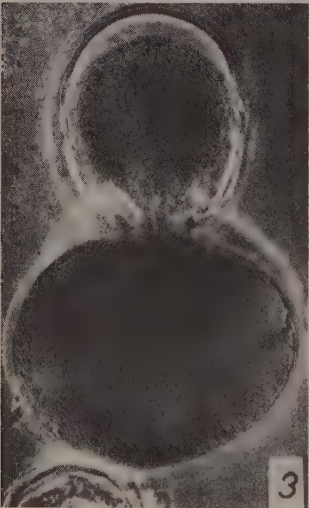
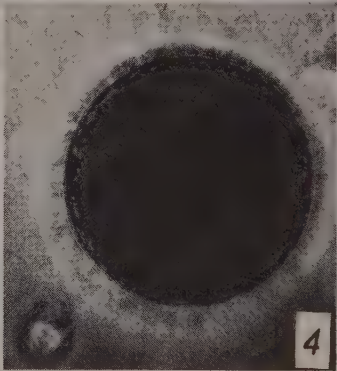
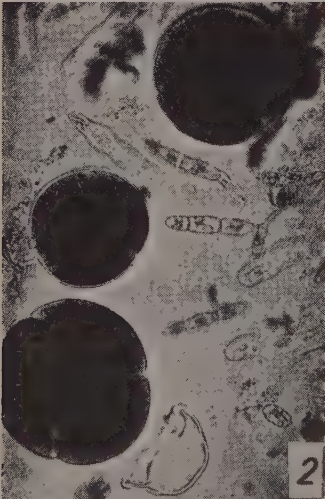
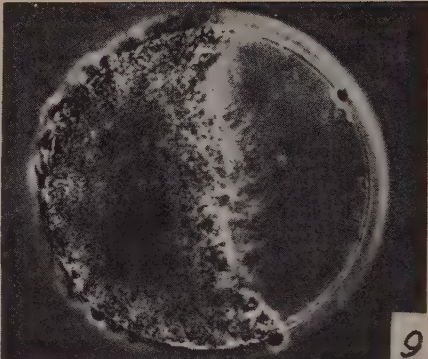
The eggs are formed in oogonia in a number of 8 or 4. Besides oogonia — and in monoecious species also spermatangia — and different kinds of hairs the conceptacles also contain large quantities of mucilage, which is important for the release of the eggs. The oogonia also contain mucilage between the eggs and the oogonial wall (the exochite). Many fucoids are

intertidal species. At low tide they are exposed to the air and evaporation takes place. When submerged again water is absorbed and the mucilage swells. This swelling causes the ripe oogonia to burst. This mucilage swelling mechanism also forces the eggs and even mature oogonia through the ostiole of the conceptacles and the eggs are released.

The eggs of *Fucus* and related genera have usually been described as naked, spherical bodies of protoplasm, containing i.a. numerous chromatophores. In an ordinary microscope no cell-wall or any kind of membrane around the eggs can be observed. However, by using special methods the morphology and submicroscopical structure of the eggs can be studied more accurately.

One of the first questions is whether or not the mature eggs are covered with a wall. In hypertonic sea water, they shrink considerably but no cell wall becomes visible. As a rule a mixture of equal parts sea water and 2.5 N NaCl was used in these experiments. It must further be pointed out that the surface remained smooth, despite the shrinkage of the fertilized egg. The only observable change in the eggs was a reduction in size. A wall of firm construction can thus not exist. When observed through a phase contrast microscope the surface layers of the eggs are quite well delineated (Figure 4). The eggs are surrounded by a gelatinous coat, tangentially stratified at least in the inner layers. Under this gelatinous coat a very thin, but distinct layer is visible. This layer, which encloses the cytoplasm, was earlier designated as the egg membrane (Levring 6). The outermost part of the cytoplasm probably consists of a lipo-protein plasma membrane, but it cannot be directly distinguished in the microscope. In the peripheral part of the cytoplasm a more or less diffuse cortical layer can be observed. This designation is perhaps more correct than »layer of cortical granules», which was used earlier (cf. Levring 6), as proper granules cannot be distinguished. The gelatinous coat can also be seen quite well in dark field.

If distilled water is added to the eggs, they swell and disintegrate. During this process several interesting observations can be made. Of the species used for these experiments *Ascophyllum* and *Fucus serratus* were found to constitute the best material. In *Fucus vesiculosus* the phenomena were less easily observable. The volume of the eggs increases as water is absorbed. Very soon a number of blisters are formed on the surface of the eggs (Figures 5—9). The gelatinous coat dissolves and the egg membrane can be distinguished very plainly, especially where the blisters are formed (Figures 7—9). A picture like this can only be seen for a very few seconds, then a smaller or greater number of the blisters burst, forming holes in the egg membrane; there is an immediate contraction of the egg surface and cytoplasm is forced through the holes. The addition of distilled water seems to



be a very simple way to demonstrate the occurrence of an egg membrane. Especially *Ascophyllum* and *Fucus serratus* gave some very good pictures. When taking up water the eggs of these species sometimes gave the impression of having one half filled with water and the other half containing the cytoplasm (Figures 8—9). The egg membrane and the gelatinous coat, which soon is washed away, are seen very plainly. One gets the impression that the egg membrane has elastic properties. Upon contraction of this membrane, the cytoplasm, which has taken up water, is forced through the breaks, where the egg membrane often forms small funnels. In a few minutes, however, the eggs are entirely disintegrated.

In a polarizing microscope the surface shows a weak birefringence, positive in the radial direction. This means that the surface must contain rod-shaped molecules arranged perpendicularly to the surface of the egg. This birefringence is most likely due to the occurrence of lipoids in the lipo-protein plasma membrane, as it disappears if the eggs are treated with distilled water. Hypotonicity has the same effect on the birefringence of the chromoplasts, which also contain lipoids. Sometimes it can be seen that the cortex after this treatment with distilled water exhibits a very weak birefringence. But this is now negative, probably due to the occurrence of tangentially arranged protein molecules (cf. Levring 6). The reason why this negative birefringence cannot be observed before the treatment with distilled water is obviously that the birefringence of the lipoids, which was positive, is too strong compared with that of the proteins. This weaker birefringence is thus impossible to distinguish as long as the lipid molecules are not washed away by the water.

According to Levring (6) the viscosity of the cytoplasm is lower in mature, unfertilized eggs than in fertilized ones. Eggs of *Ascophyllum* were studied in a centrifuge microscope. A distinct stratification could be observed after 4 minutes at a speed corresponding to the acceleration 7000 g. The eggs were also elongated (cf. Figure 10 A—C). The stratum (a), which obviously contains oil, was soon lost (Figure 10 C) from the centripetal part of the egg.

Figure 1. *Ascophyllum nodosum*, oogonia.

Figure 2. *Ascophyllum nodosum*, oogonia. Below a group of 4 eggs, which have left the oogonium.

Figure 3. *Ascophyllum nodosum*, eggs leaving an oogonium.

Figure 4. *Fucus serratus*, mature egg. Phase contrast.

Figure 5 and 6. *Fucus vesiculosus*, egg treated with distilled water. Phase contrast.

Figure 7. *Fucus serratus*, eggs treated with distilled water. Phase contrast.

Figure 8. *Fucus serratus*, egg treated with distilled water. Phase contrast.

Figure 9. *Ascophyllum nodosum*, egg treated with distilled water. Phase contrast.

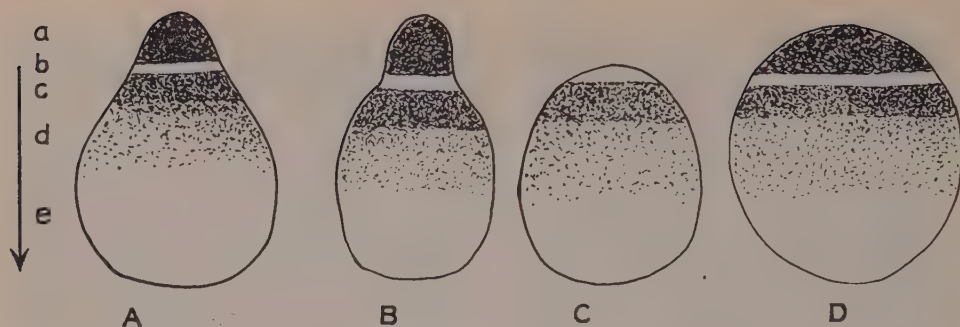


Figure 10. A—C. *Unfertilized eggs studied in the centrifuge microscope.* In C the layer a is lost after about 4 minutes. — D. *Fertilized egg.* — a dark brown layer (=oil), which is getting lost in unfertilized eggs; b colourless (water); c brown, containing chromoplasts; d yellow, continuing to e colourless (nearly). There is a sharp limit between c and d in fertilized eggs, where the wall and the layer d is stained by toluidine blue.

The egg membrane is thus not strong enough to prevent the egg from being divided.

Attempts have also been made to study the surface layers with various chemical reagents. As has been shown earlier (cf. Levring 6, 7) a treatment of the eggs for about one hour with comparatively strong solutions of trypsin (0.5—0.1 per cent) affects the egg membrane in such a way that no fertilization membrane is formed after fertilization. It is thus likely that protein components are dissolved and the mechanism of the cellwall formation has been disturbed. From this experiment it may be assumed that the egg membrane contains protein components. This assumption, however, cannot be regarded as proved, especially as a commercial preparation of trypsin was used.

The mature eggs are not stained by toluidine blue. But the slime which is released with the eggs from the oogonia is stained very strongly metachromatically. This reaction shows the presence of polysaccharide sulphates. As fucose sulphate, also known as fucoidin, occurs commonly in the cell walls of the phaeophyceae, it seems most likely that the metachromatically stainable substance consists at least partly of fucose sulphate. Shortly after the liberation of the eggs from the oogonia they are slightly stainable on the surface. This is probably due to slime attached to the surface, which is soon dissolved in the surrounding water and the reaction with toluidine blue becomes negative.

As shown in the diagram (Figure 11 A) the surface of a mature, unfertilized egg consists of the following layers: 1) A gelatinous coat, tangentially strati-

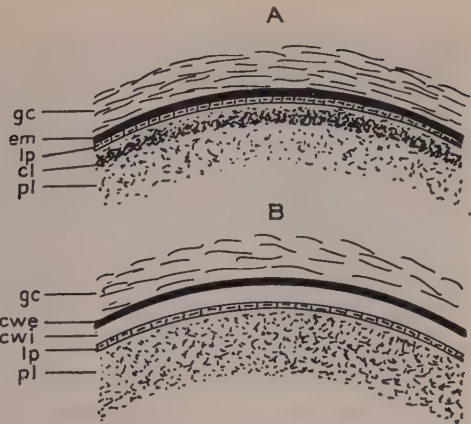


Figure 11. *Diagram of the egg surface.* — *A* Mature, unfertilized egg. *B* Fertilized egg. — *gc* gelatinous coat; *em* egg membrane; *lp* lipo-protein membrane; *cl* cortical layer; *pl* cytoplasm; *cwe* cell wall, outermost part; *cwi* cell wall, innermost part.

fied (*gc*); 2) the egg membrane, of a more or less unknown chemical composition but very likely containing protein components (*em*); 3) the lipo-protein plasma membrane (*lp*), and 4) the cortical layer (*cl*).

The spermatozooids

The general morphology and shape of the spermatozooids is well known. They are pyriform, with two cilia situated on the side near the eye-spot. The cilia are of different length, the one turning forward being the shorter one. The spermatozooids are released from the spermatangia with slime, which like the slime of the ogonia and the walls of the spermatangia are stained metachromatically by toluidine blue. Hotchkiss' reaction is also positive. The slime and the walls thus contain polysaccharide sulphates, probably fucoidin.

The spermatozooids were studied with phase contrast optics (Figures 12—13). It was then found that they were surrounded by a thin gelatinous coat, which, like a part of the cell content of the spermatozoid itself, was stained metachromatically by toluidine blue and which also gave a positive Hotchkiss' reaction. The spermatozooids are thus covered with a thin gelatinous coat containing polysaccharide sulphates, most likely fucoidin.

It is of course very difficult to settle the question of the rôle of this coat, especially during the fertilization process. Also, the spermatozooids are so small that a detailed study of the coat is impossible. If the spermatozooids are kept in sea water for some hours, however, the toluidine blue reaction often seems to become weaker. The stainable substance would thus seem to have been dissolved by the sea water. It was also investigated whether spermatozooids which had been swarming for some time and where the stainable

coat obviously at least partly had been dissolved by the water still were able to fertilize eggs or not. No difference could be observed. Apparently the polysaccharide sulphates of the spermatozoid surface seems to have no direct influence on the fertilization.

It is interesting to note that a similar gelatinous coat has been found on the gametes of the chlorophyceae *Ulva lactuca* (cf. Levring 8).

Fertilization and formation of the fertilization membrane

At fertilization the spermatozooids swarm around the egg for a variable length of time (up to about half an hour), one of them penetrates into the egg and the rest become sluggish and soon die. According to Cook et al. (2) egg water from both *Fucus serratus* and *vesiculosus* attract spermatozooids not only of these two species equally well but also spermatozooids of *F. spiralis*. This lack of specificity of the chemotactic substances was further confirmed by observations on the eggs themselves. Recently Cook and Elvidge (3) have shown that a number of hydrocarbons, ethers and esters in dilute solutions in sea water have true chemotactic properties for the spermatozooids of *Fucus serratus* and *vesiculosus*. The chemotaxis is therefore not the unique property of one specialized substance. The active substances were by these authors found to be chemically unreactive compounds, of molecular weight 70—120, and consist of molecules containing straight chains of 5—6 atoms.

Very soon after fertilization a membrane becomes visible. It is best observed with phase contrast optics (Figure 14). After about 10 minutes the wall is strong enough not to burst if the egg is put into distilled water. The thickness of the membrane increases rapidly (Figure 15). If treated with hypertonic sea water the eggs are plasmolysed and the fertilization membrane is seen very plainly. The plasmolysis is angular (Figures 16—17).

When examined with a polarizing microscope the wall of the eggs shows a very strong birefringence (Figure 18). A close examination in the phase contrast microscope shows that the wall consists of two different layers. The outermost one is a remnant of the egg membrane and is still covered by the gelatinous coat. It is the inner part, which increases in thickness, which shows a very strong birefringence, negative in the radial direction. Therefore, it is this part of the fertilization membrane which contains rod- or foil-like molecules arranged tangentially to the surface of the egg. The interior layer (especially its peripheral part) was stained blue by zinc chloride-iodine mixture. It obviously contains cellulose, which substance also causes the very strong birefringence, seen very soon after fertilization. Finally the cortical layer seems to disappear gradually.

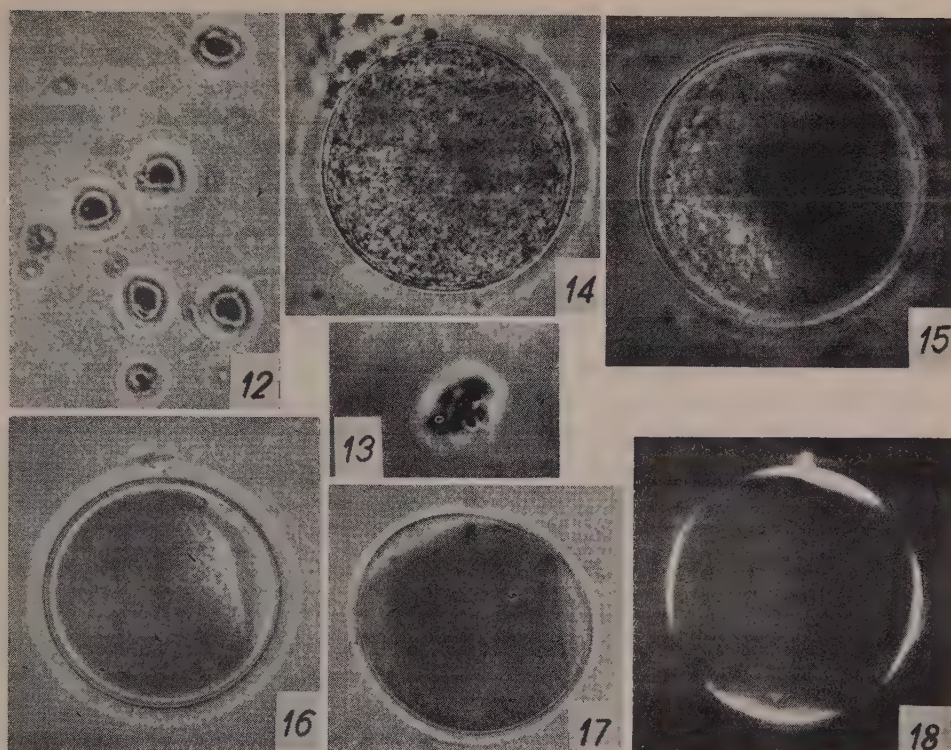


Figure 12. *Ascophyllum nodosum*, spermatozooids. Phase contrast.

Figure 13. *Fucus serratus*, spermatozoid. Phase contrast.

Figure 14. *Ascophyllum nodosum*, fertilized egg. Phase contrast.

Figure 15. *Ascophyllum nodosum*, fertilized egg, older stage. Phase contrast.

Figure 16. *Fucus vesiculosus*, fertilized egg, plasmolysed. Phase contrast.

Figure 17. *Fucus edentatus*, fertilized egg, plasmolysed. Phase contrast.

Figure 18. *Ascophyllum nodosum*, fertilized egg in polarized light.

Like unfertilized eggs the fertilized ones were stained with toluidine blue. The peripheral parts of the cytoplasm as well as the inner part of the wall are stained metachromatically. The reaction gradually becomes stronger after fertilization. Hotchkiss' reaction was also positive for the same parts of the egg, which were stained by toluidine blue. This staining is obviously due to the occurrence of polysaccharide sulphates, and, as mentioned above, most likely fucoidin. As the staining is in the cortical layer and the inner part of the wall the most likely explanation is that the substance is released in the cortical layer and transported through the plasma membrane to the wall, where it becomes one of the constituents.

Fertilized eggs were also studied with the centrifuge microscope. The viscosity was, as mentioned earlier in this paper, higher than in mature, unfertilized ones. A plain stratification was obtained after about 6 minutes at a speed corresponding to the acceleration 9—10,000 g. The wall was never broken. Compared with unfertilized eggs (Figure 10) the border line between c and d was much sharper. After centrifuging the layer d and the inner part of the wall could be stained metachromatically with toluidine blue (Figure 10 D).

If *Fucus* and *Ascophyllum* eggs were treated with comparatively strong solutions of trypsin (Merck, commercial preparation): 0.5—0.1 per cent for 4—12 hours, hardly any visual change could be observed. The eggs were washed with sea water and a suspension of spermatozooids added. The eggs seemed to be attacked in a normal way or perhaps a little less actively than is the case with untreated eggs. No wall was formed, however. In a few cases a nuclear division seemed to have taken place after 12—24 hours. Of interest is that the peripheral parts of the eggs, which were obviously fertilized, were metachromatically stained by toluidine blue, even though the staining was a little weaker than in normal fertilized eggs. No activation of unfertilized eggs with very weak trypsin solution (0.05—0.001 per cent) was obtained, even if the content of Ca^{2+} -ions was increased (cf. Bohus-Jensen 1; Levring 7).

The respiration of unfertilized and fertilized eggs has been studied by Whitaker (16). The oxygene consumption of fertilized eggs (*Fucus vesiculosus*) was found by him to be 190 per cent of that of unfertilized eggs. The oxygen consumption was determined by the Warburg method. In similar experiments I have been able to find a considerable increase in oxygen consumption after fertilization. The increase varied in different experiments, the oxygene consumption after being between 170 and 190 per cent of that before fertilization. In my experiments the effect of dupunol was also studied. It is interesting to note that dupunol increased respiration nearly as much as fertilization does (Table 1). But, as in Whitaker's experiments it was found that a very great number of the unfertilized eggs were shaken to pieces during the experiments, thus making the determinations more or less unreliable.

The blocking effect of dupunol on the fertilization has been shown earlier in experiments with the Australian species *Hormosira Banksii*. Dupunol is a mixture of sulphonates of long-chain aliphatic alcohols differing only in the length of the hydrocarbon chains (cf. Putnam 9). The experiments were repeated with *Ascophyllum* and the *Fucus* species. The following dilutions in sea-water of the substance were used: 0.004, 0.002, 0.001, 0.0005, and 0.00025 per cent. Ripe spermatangia and mature eggs were put into the dif-

Table 1. *Respiration of eggs of Fucus vesiculosus in a Warburg experiment.*
Temperature 15°.

Time minutes	Oxygene consumption ($\text{mm}^2 \text{O}_2$) of egg suspension		
	Unfertilized	Fertilized	Unfertilized + 0.001 % dupunol
20	0.9	2.6	1.4
40	2.7	6.2	4.1
60	5.6	10.8	8.7
80	8.0	15.1	12.6
100	11.1	20.0	17.5
120	13.5	24.5	21.8
140	16.2	28.9	26.1

ferent solutions. The spermatozooids were immediately liberated. In the 0.004 per cent solution they soon lost their mobility. In 0.002 per cent they swarmed for a few minutes. No fertilization was observed. In the 0.001 and 0.0005 per cent solutions the spermatozooids were liberated and swarmed in a quite normal way but they never attacked the eggs, which were never fertilized. In 0.00025 per cent a normal fertilization took place. These results are in accordance with the results obtained in experiments with *Hormosira*, mentioned above.

It may be of interest to recall the behavior of the spermatozooids during normal fertilization in pure sea water. At first they swarm around the eggs for a time of variable length. One penetrates the egg and the rest soon become sluggish and remain motionless near the egg. If there are very few spermatozooids present they may swim away instead. It can also be observed that in a drop of sea water where a number of eggs has been fertilized all the spermatozooids soon lose their motility. In pure sea water without any eggs they swarm for a long time.

It therefore seems very likely that a substance acting like dupunol is released from the egg at the same moment as the latter is penetrated by the spermatozoid. Consequently the remaining spermatozooids lose their mobility and further fertilizations are blocked. The substance suggested may therefore be connected with the mechanism which prevents polyspermy. Analogies with the conditions at the fertilization of sea-urchin eggs are striking (cf. Runnström, Tiselius, and Lindvall 12, 13; Runnström and Lindvall 11).

Discussion and Summary

The properties of eggs and spermatozooids of some Fucoids have been studied. It was found that the spermatozooids are surrounded by a thin gelatinous coat containing polysaccharide sulphates (probably fucoidin). The

same reaction as that of the coat, which seems to be partly dissolved by the water, is given by the slime, which is released from the spermatangia, together with the spermatozooids, and the walls of the spermatangia.

The surface of the mature, unfertilized eggs consists of the following layers (Figure 11 A): 1) a gelatinous coat stratified tangentially which seems to be partly dissolved by water; 2) an egg membrane, a very thin layer, which plays an essential rôle in the formation of the fertilization membrane; 3) a lipo-protein plasma membrane; 4) an innermost cortical layer, which produces material for building up the cell wall after fertilization.

After fertilization a cell wall is soon visible. It consists of two layers (Figure 11 B): 1) the innermost is strongly (negative in radial direction) birefringent and must therefore contain rod-shaped molecules arranged tangentially to the surface of the egg; it contains cellulose and polysaccharide sulphates (fucoidin); the outermost is a remnant of the egg membrane covered with the gelatinous coat. The peripheral part (the cortical layer) of the cytoplasm also contains polysaccharide sulphates. The viscosity is higher in fertilized eggs than in mature, unfertilized ones. The cytoplasm granules of unfertilized eggs seems to have a somewhat finer structure than those of the fertilized ones.

It is obvious that the cell wall formation after fertilization involves a number of reactions in or just under the surface and various enzyme systems are thereby released. The effect of trypsin and dupunol on the fertilization and on the cell wall formation is very interesting. The trypsin seems to affect the egg membrane in such a way that no cell wall can be formed. The dupunol molecules block the surface of the egg so that the spermatozooids are not able to react with the egg. Most probably lipoids are replaced by the dupunol. This reagent also increases respiration considerably. At fertilization, cell wall material is released from the periphery of the egg mainly in the cortical layer and is forced out through the plasma membrane. In some cases slight contractions in the surface have been observed, which give further support to this explanation. This material reacts with the egg membrane which acts as a kind of templete supporter and the new wall is formed on the interior side of this membrane. The new wall is very strong, contains cellulose and polysaccharide sulphates (probably fucoidin). It contains also most likely alginic acid, but there is no method known as yet which makes it possible to prove this assumption. It must also be pointed out that the cell wall formation starts immediately after the penetration of the spermatozooids into the egg and long before any nuclear activity can be observed. The formation of the cell wall can therefore hardly be a result of a nuclear action. After fertilization the respiration of the egg also increases considerably.

The agreement with the conditions in the sea urchin eggs is very striking (cf. Runnström 10). The structure of the surface seems to be nearly the same. The cortical layer of the *Fucus* eggs corresponds to the layer of true cortical granules in sea urchin eggs. After fertilization these granules are forced through the lipo-protein membrane and take part in the formation of the fertilization membrane on the inner side of the vitelline membrane, which more or less corresponds to the egg membrane of the *Fucus* eggs. The mechanism of the fertilization membrane formation thus seems to agree strikingly in these quite different groups of organisms. Also a good agreement in various details seems to exist.

The experimental part of this paper has been carried out at the Marine botanical institute, Göteborg, and at the Zoological station of the Academy of science at Kristineberg in Bohuslän.

References

1. Bohus-Jensen, A.: Activation of unfertilized sea urchin eggs by trypsin. — *Nature* 162: 931. 1948.
2. Cook, A. H., Elvidge, J. A. & Heilbron, Sir Ian: Fertilization including chemotactic phenomena in the *Fucaceae*. — *Proc. Roy. Soc., B.* 135: 293. 1948.
3. Cook, A. H. & Elvidge, J. A.: Fertilization in the *Fucaceae*: Investigations on the nature of the chemotactic substance produced by eggs of *Fucus serratus* and *F. vesiculosus*. — *Ibidem* 138: 97. 1951.
4. Farmer, J. B. & Williams, J. L.: Contributions to our knowledge of the *Fucaceae*: their life-history and cytology. — *Trans. Roy. Soc., B.* 189: 623. 1898.
5. Kniep, H.: Beiträge zur Keimungs-Physiologie und -Biologie von *Fucus*. — *Jahrb. wiss. Bot.* 44: 635. 1907.
6. Levring, T.: Remarks on the surface layers and the formation of the fertilization membrane in the *Fucus* eggs. — *Meddel. Göteborgs bot. trädg.* 17: 97. 1947.
7. — Fertilization experiments with *Hormosira Banksii* (Turn.) Dcne. — *Physiol. plantarum* 2: 45. 1949.
8. — Unpublished.
9. Putnam, F. W.: The interactions of the proteins and synthetic detergents. — *Adv. Protein Chem.* 4: 79. 1948.
10. Runnström, J., The mechanism of fertilization in metazoa. — *Adv. Enzymology* 9: 241. 1949.
11. Runnström, J., & Lindvall, S.: The effect of some agents upon the reaction of *Echinocardium* spermatozoa towards egg-water. — *Arkiv Zool.* 38 A nr 10. 1946.
12. Runnström, J., Tiselius, A., & Lindvall, S.: Gamones from the sperm of sea urchin and salmon. — *Nature* 153: 285. 1944.
13. — — — The action of androgamone III on the sea-urchin egg. — *Arkiv Zool.* 36 A nr 22. 1945.
14. Thuret, G.: Recherches sur la fécondation des *Fucacées*. — *Ann. sc. nat. S.* 4: 2. 1854.
15. Whitaker, D. M.: Physical factors of growth. — *Growth Suppl.* 75. 1940.
16. — On the rate of oxygene consumption by fertilized and unfertilized eggs. I *Fucus vesiculosus*. — *J. gen. physiol.*, 15. 1932.

The Free Amino Acids in the Leaves, Roots, and Root Nodules of the Alder (*Alnus*)¹

By

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An intensive fixation of atmospheric nitrogen takes place not only in the root nodules of leguminous plants but also in those of the alder (Virtanen and Saastamoinen, 24). For instance, in a quartz sand culture without any combined nitrogen, a nodulated *A. glutinosa* grew 261 cm in length during the second year, the simultaneous increase of nitrogen in the plant being about 6.5 g, of which considerably more than 3 g were found in the leaves (Virtanen and Saubert-v. Hausen, 25).

In order to gain information on the accumulation of soluble nitrogen compounds in the nitrogen-fixing root nodules of legumes and the alder and also on the fluctuations in the relative proportions of amino acids present during the growth period, we have determined the content of free amino acids in the root nodules of these plants. Simultaneously we have also compared the quantities of free amino acids present in the nodules with those quantities present in other parts of the plants, in order to obtain, if possible, a deeper insight into the nitrogen metabolism of these plants in general.

The free amino acids were extracted immediately after harvest from the parts of the plants under study using 70 per cent by weight ethanol. They were determined visually by comparing the spots on paper chromatogram with a reference series made under the same conditions by known amounts of amino acids. This so-called reference series technique (Polson, 13) gives

¹ A preliminary note on the main results of this investigation has been published in *Nature* (London) 170: 283, 1952.

an accuracy of within 20—30 per cent, providing that the quantity of amino acids is suitable (2—30 μ g) and the shape of the spots is good. If such an accuracy is desired for each amino acid, several chromatograms have to be made from samples containing different amino acids in widely varying concentrations (Lindan and Work, 11). We have come to the same conclusion. Each of the values recorded in this work is based on at least 3 chromatograms which had suitable spots for comparison (See table 1, last column). Most of the values are correct with a maximal error of 20—30 per cent. The error may be somewhat higher in certain cases, with arginine, ornithine and γ -amino butyric acid, which often produce spots of irregular shape; yet it is always within the limits —50 per cent and +100 per cent.

The values obtained with this semiquantitative technique are only approximate, yet definite conclusions can be drawn from them when the differences are many times greater than the maximum error.

This paper reports results obtained with grey alder *Alnus incana* and black alder *A. glutinosa*. The results obtained with the leguminous plants will be reported separately (Virtanen et al., 22).

Results

The semiquantitative results, calculated upon dry weight, are summarized in Table 1.

As can be seen from Table 1 and Figures 1—8 L(+)-citrulline is the predominating free amino acid in the roots and nodules of alder. During the period May—August when the leaves are green, the nodules of both *A. glutinosa* and *A. incana* contain citrulline to the extent of about 0.2—0.3 per cent of the dry weight of the tissue. The citrulline content of both alder species increases greatly towards the autumn, until the leaves fall. So for instance, the nodules of *A. incana* harvested in October (under natural conditions leaves just fallen) contained citrulline to about 0.9 per cent of the dry wt. and the nodules of *A. glutinosa* (greenhouse culture, leaves fallen in November) nearly 2 per cent of dry wt. in January.

As can be seen from Table 1 and Figures 1—8 there is remarkable similarity, but distinct differences too, between the two species of alder in the composition of the free amino acids of the nodules. All preparations of *A. incana* (nos. 4—6) differ from the corresponding ones of *A. glutinosa* by containing large amounts of γ -aminobutyric acid (0.1—0.2 per cent of dry wt.), whilst *A. glutinosa* extracts contain but traces of it. Another difference is that aspartic acid is completely absent from *A. incana* extract in autumn whereas *A. glutinosa* extracts contain almost as much aspartic acid as glutamic

Table 1. *The free amino acids of alder tissues in different seasons when compared to a unit of dry weight. A. incana samples taken from nature, A. glutinosa samples from a greenhouse.*

Prep. No	Part of plant	Harvested	μg amino acid / 100 mg dry weight										Fig. No	N:o of chrgs made
			Citr	γ -AB	Arg	Orn	Glu	Asp	Ala	Ser	Thr	Val		
			33	29	14	23	17	16	2	8	9	3		
Alnus incana:														
1	leaves	June 3	46	9	9	—	26	10	8	9	12	4	—	5
2	stem	"	35	2	3	—	2	1	2	2	1	1	—	4
3	roots	"	151	3	4	—	6	4	5	2	2	2	—	4
4	nodules	"	300	93	2	—	17	6	17	4	2	5	—	4
5	"	Aug. 1	170 ¹	190	—	—	30	—	—	—	—	—	1	6
6	"	Nov. 6 ²	870	160	—	—	42	—	—	—	—	—	2	4
Alnus glutinosa:														
7	roots	May 28	70	20	20	—	16	3	10	2	—	—	4	3
8	nodules	"	180	10	20	—	25	2	16	3	—	—	3	3
9	sterile roots ³	Jan 22 ²	100	3	—	—	10	10	3	1	—	—	8	3
10	roots far from nodules	"	250	3	35	50	20	10	15	7	2	—	7	5
11	roots near to nod.	"	900	10	200	300	60	30	20	10	5	—	6	6
12	nodules	"	1800	10	10	20	60	60	15	3	1	—	5	7

¹ Also determined photometrically with diacetylmnonoxime with the result 166/100 mg dry wt.

² The leaves fallen.

³ Plants grown in medium containing combined nitrogen.

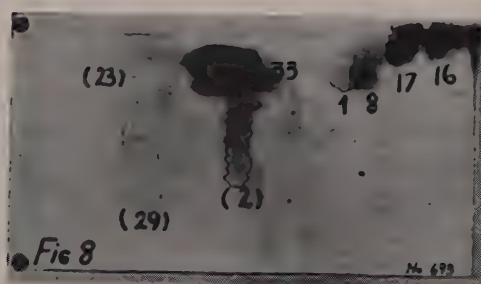
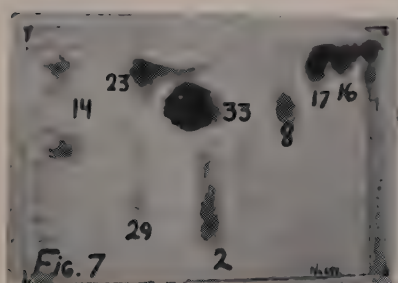
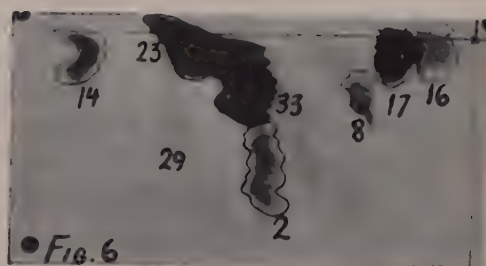
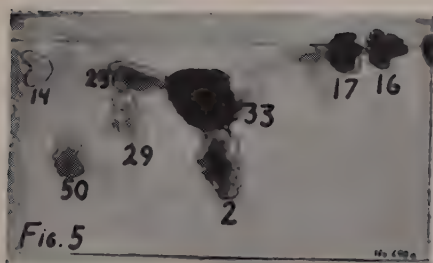
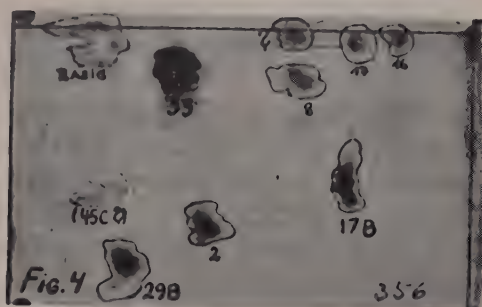
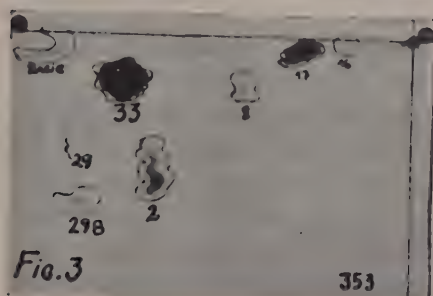
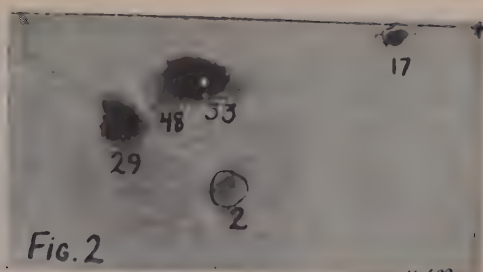
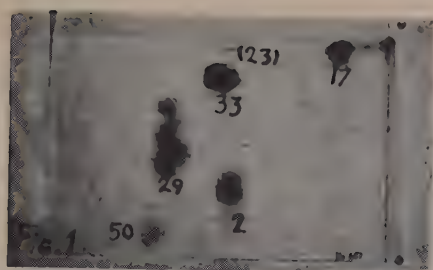


Figure 1. Nodules of *A. incana*, August.

Figure 2. Nodules of *A. incana*, October.

Figure 3. Nodules of *A. glutinosa*, May.

Figure 4. Roots of *A. glutinosa*, May.

Figure 5. Nodules of *A. glutinosa*, January.

Figure 6. *A. glutinosa*, roots from the neighbourhood of the nodules, January.

Figure 7. *A. glutinosa*, roots farther from the nodules, January.

Figure 8. *A. glutinosa*, uninoculated roots, January.

acid. The latter also contains in summer and winter alike some arginine and ornithine. Both *Alnus* species contain in summer an unknown compound (50) which is none of the compounds presented in Figure 12 (map of amino acids). Preparations No. 4 and 8 obtained from the nodules harvested in May (*A. glutinosa*) and June (*A. incana*) also contained some tyrosine, valine, and leucine and/or isoleucine, none of which were found in preparation No. 5—6 and 12 made in winter. In certain cases a faint ethanolamine spot (Figure 4) was apparently obtained from *A. glutinosa* extract although this has not yet been identified for certain.

The roots of *A. glutinosa* (Figure 6 and 7) taken from a greenhouse culture in January differ from the nodules taken from the same plant (Figure 5) especially by containing much more arginine and ornithine (see also Table 1). The parts of roots in the nearest vicinity of the nodules contain about 0.2 per cent arginine and about 0.3 per cent ornithine of the dry weight, those farther from the nodules only about $\frac{1}{5}$ of these amounts. The same seems to apply also to other amino acids. There seems to be no qualitative difference in the amino acid composition of different parts of the roots.

The stem and the leaves of *A. incana* (Table 1, prep. No. 1 and 2) do not differ qualitatively from the roots of the same plant. There are remarkable quantitative differences, however. The leaves (prep. No. 1) differ essentially from the roots by containing much less citrulline but about 2—6 times more of other amino acids. When compared with the stem, the leaves contain appr. 4—10 times more of all amino acids, excepting citrulline, of which they only contain appr. the same amount.

Unnodulated roots

In order to ascertain whether the citrulline in the nodules of alder is possibly a product of nitrogen fixation or whether it is characteristic of the general nitrogen metabolism of roots, the free amino acid composition of unnodulated alder roots was also examined. These alders were grown in sand and water cultures and supplied with nitrate, ammonium and aspartic acid nitrogen. It was found that such roots also contain citrulline (Figure 8 and prep. No. 9, Table 1) although in much smaller quantities than do the nodulated roots (only about 0.1 per cent of dry weight in winter). Hence, the presence of citrulline in the alder nodules is not due to the fixation of molecular nitrogen in them.

Nitrogen fractions of the alder

Since it was of interest to know the amount of citrulline expressed as a fraction of total and soluble nitrogen, these values were determined in *A. in-*

Table 2. Nitrogen fractions in the different parts of *Alnus incana*. Samples taken from nature in July.

Parts	Dry weight % of fr. wt.	Total-N % of dry wt.	Ethanol- sol. N, % of		Citrulline-N, * % of			NH ₃ -N, % of		
			dry wt.	tot.-N	dry wt.	tot.-N	sol. N.	dry wt.	tot.-N	sol. N.
leaves	27.9	3.22	0.068	2.12	0.011	0.34	16.2	0.0013	0.040	1.91
stem	58.2	0.64	0.033	5.16	0.008	1.25	24.2	—	—	—
roots	55.1	1.09	0.197	18.10	0.036	3.30	18.3	—	—	—
nodules...	24.3	2.69	0.308	11.45	0.072	2.68	23.4	0.0039	0.145	1.27

* Citrulline figures are based upon semiquantitative chromatographic determinations.

cana in June, all samples being taken from one plant (see Table 2). The total nitrogen is highest in the leaves and lowest in the wooden stem. Ethanol soluble nitrogen is astonishingly low in the leaves, when compared with the other parts of the plant. The nitrogen fixed in the nodules in the symbiosis is evidently rapidly passed on, since the soluble nitrogen of the roots is much higher than that of the nodules when calculated as percentage of total N of the tissue.

Citrulline-N, like the soluble N, shows great differences between the different tissues, when compared with the total-N, being lowest in the leaves and highest in the roots. However, when the citrulline-N is calculated as a fraction of the soluble N, no sharp differences are to be noticed. The differences are then of the same degree as the error in the chromatographic citrulline determinations.

Citrulline-N corresponds only to ca. 20 per cent of the soluble N. Since, on the basis of Table 1, all the other amino acids together correspond to a still smaller percentage, the nature of the bulk of the soluble nitrogen is still obscure. It is not mainly due to peptides although a small amount of peptide nitrogen is evidently present, for after hydrolysis of the ethanol extract the amounts of alanine and glutamic acid are appr. doubled.

For comparison, free ammonia was determined in the leaves and nodules using fresh material (for details, see experimental part). Although care was taken to avoid splitting of ammonia from labile amino compounds, this may have happened to some extent.

Isolation of L(+)-citrulline as Cu-salt

As citrulline was proved to be a very noticeable component of the soluble nitrogen in the alder nodules we considered it important to confirm its identity by a method other than paperchromatography. For this purpose citrulline was isolated from the root nodules of alder as the Cu-salt and characterized in the following manner:

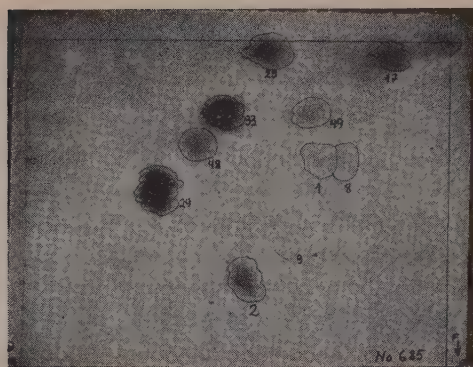


Figure 9. Preparation no. 6 after removal of the bulk of citrulline.

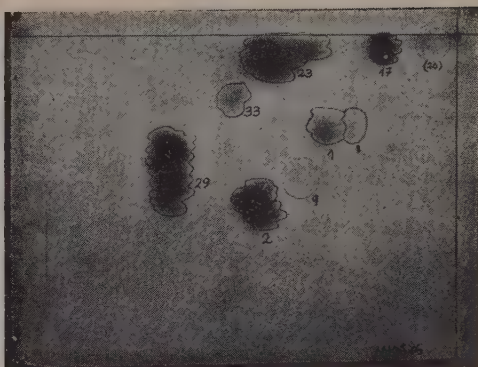


Figure 10. Preparation of figure 9 after hydrolysis by HCl.

The nodules of grey alder (*A. incana*) harvested in nature in October 1951 (prep. no. 5) were extracted using 70 per cent by wt. ethanol, the ethanol was evaporated in vacuo and the precipitate was dissolved in water. A brown, rapidly darkening solution was obtained containing amino acids but being also rich in other compounds, which were slowly polymerized, for a dark-brown precipitate was continuously formed for several weeks, thus making difficult the treatment of the extract. Amino acids were therefore purified from these impurities by ion exchange in a manner described in detail in the experimental part of this paper. The high purity amino acid solution was treated with CuO, whereupon a light-blue, difficultly soluble Cu-salt was crystallized. After recrystallization the salt melted at 266—268° C when rapidly heated, but when slowly heated it was carbonized without melting at 252,5 C°, and it proved to be in every respect identical with the authentic Cu-L(+)-citrullinate.

The yield was 0.6 mg/100 mg dry weight (raw product), which corresponds to 0.43 mg free citrulline/100 mg dry weight. By paperchromatography (Figure 9) it was detected that a considerable part of citrulline still remained uncrystallized in the mother liquor, this amount corresponding to about 0.2—0.3 mg/100 mg dry weight. Consequently, the nodules contained altogether about 0.7 mg citrulline per 100 mg dry weight. This is a slightly lower value than the one obtained by the paper-chromatographic method direct from the initial extract (0.87 mg/100 mg dry wt.).

γ-amino butyric acid

Since γ -amino butyric acid was found in abundance in the nodules of *A. incana* — in August it was the predominating free amino acid — an attempt was made to



Figure 11. *Citrulline partially decomposed in HCl-hydrolysis.*

confirm its identity as accurately as possible. It was found by paperchromatography (see Figure 12) that γ -amino butyric acid travels as hydrochloride at a higher speed (29 B) than under fully neutral conditions in butanol. Even a small amount of HCl contained in a sample drop is sufficient to produce the spot 29 B. Often both spots are visible at the same time and then 29 B is usually followed by a tail which sometimes connects both spots. This occurrence of two spots first caused confusion, but as soon as it was explained by means of detailed study at different pH it has been of great advantage in the identification of γ -amino butyric acid. The sensitive dependence of the R_F -value of γ -amino butyric acid on pH was first shown by Synge (19) in a paper which was published while the present work was in progress. In that paper Synge also introduced the possibility of isolating electro dialytically γ -amino butyric acid from neutral amino acids at a suitable pH (slightly below pH 4), when γ -amino butyric acid travels to the cathode cell whilst the neutral amino acids almost completely remain in the middle cell. Our preliminary experiments by partition chromatography using paper columns had shown that it is possible to isolate γ -amino butyric acid from alanine and citrulline using water-saturated phenol as solvent, yet the separation was incomplete even with long columns owing to the small differences in the R_F -values. Further, our experiments showed that the evaporation residue contained brown impurities, possibly oxidation products of phenol, which prevented crystallization of γ -amino butyric acid. We therefore decided to isolate γ -amino butyric acid electro dialytically by the method of Synge. The electro dialysis was carried out with preparation no. 6 — after the bulk of citrulline had been removed as the Cu-salt — by maintaining the pH of the middle cell at about pH 4 by means of HCl. A very good separation was effected since we found paperchromatographically that γ -amino butyric acid was in this preparation the only ninhydrin-positive compound which had travelled to the cathode cell, whilst citrulline and alanine had remained almost completely in the middle cell. The catholyte contained, however, some impurities because γ -amino butyric acid could not be crystallized from it in spite of repeated attempts. These impurities could not be removed by treatment with active carbon and the small quantity of the substance made more extensive manipulations impossible. Other investigators, too, have observed that γ -amino butyric acid isolated from a natural product, is difficult to crystallize. Although we failed to completely characterize the γ -amino butyric acid isolated from the nodules of alder, its identity is practically proved by the following tests:

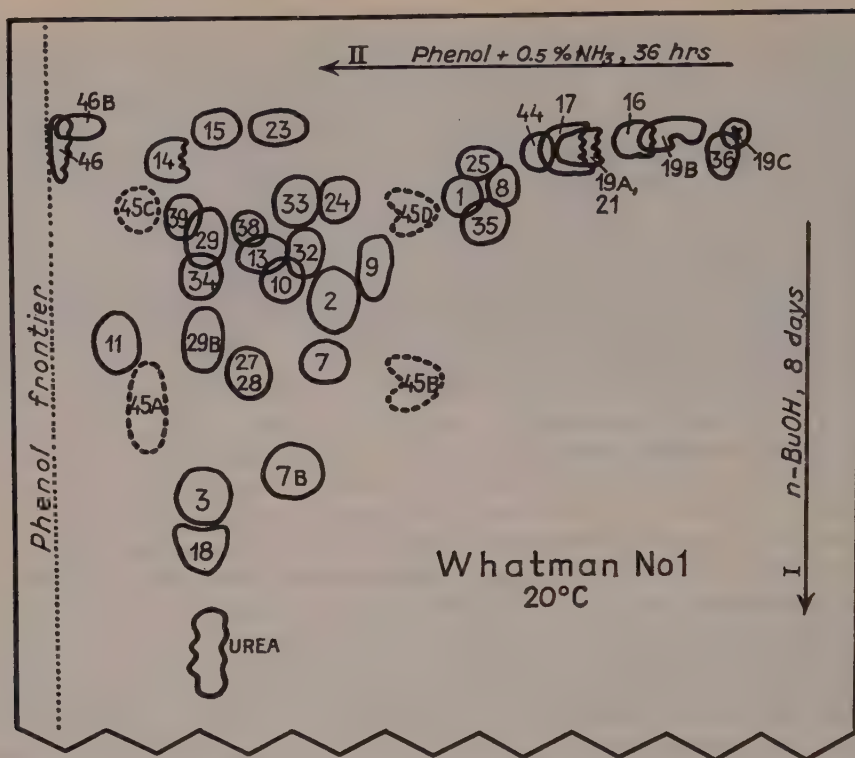


Figure 12. Map showing the positions of amino acids and urea in two-dimensional chromatograms.

1=gly, 2=ala, 3=val, 7=tyr, 8=ser, 9=thr, 10=hypro, 11=pro, 13=his, 14=arg, 15=lys, 16=asp, 17=glu, 18=met, 19 A and B=glutathione, 19 C=H₂O₂-oxidised glutathione, 23=ornithine, 24=gluNH₂, 25=aspNH₂, 27=α-amino-n-butyric acid, 28=α-amino-i-butyric acid, 29=γ-amino-butyric acid, 32=β-alanine, 33=citrulline, 34=sarcosine, 35=taurine, 36= cysteic acid, 38=methionine sulphone, 39=methionine sulphoxide, 44=ethanolamine phosphoric acid, 45 A-D=ethanolamine, 46=cadaverine, 46 B=agmatine.

- 1) When chromatographed in a mixture with authentic γ-amino butyric acid it was proved to be quite identic in all solvents (phenol, butanol, tertiary amylalcohol).
- 2) The R_f value showed sensitive dependence on pH identically with γ-amino butyric acid.
- 3) The spot did not disappear in the copper test of Crumpler and Dent (4) which shows that α-amino acid is not concerned.
- 4) Complete resistance to acid hydrolysis.

Acid hydrolysis of nodule extract

A paperchromatographic analysis of the mother liquor from preparation no. 6, which was left after the main part of citrulline was crystallized as the

Cu-salt, revealed, after precipitation of Cu as sulphide, two unknown ninhydrin-positive compounds, nos. 48 and 49, possibly peptides. No. 48 was located in the position of histidine, but was not histidine, because it gave a violet spot and disappeared on acid hydrolysis. When this preparation was hydrolyzed with hydrochloric acid (6 N, 24 hr at 108° C in a sealed tube) spots 48 and 49 disappeared and simultaneously alanine, glutamic acid, some glycine and possibly also γ -amino butyric acid were liberated (Figure 10). Citrulline was decomposed to a great extent forming ornithine (identified only by paperchromatography). This was surprising, because according to Wada (20) citrulline is decomposed to ornithine only in alkaline solution but to proline in acid hydrolysis.

For control a HCl-hydrolysis was made with pure L(+)-citrulline under the above conditions and a similar result was noted: citrulline was partly decomposed and replaced by approximately the same amount of ornithine but no proline could be found (Figure 11). This latter knowledge may be of importance when ornithine is found in the acid hydrolysates of biological preparations.

Urea-determination

In order to find out, whether the citrulline-containing extracts also contained urea and possibly other ureides besides citrulline, two-dimensional n-butanol-phenol chromatograms were prepared from extracts 1, 5, 6, and 9 to 12. These were sprayed with p-dimethyl aminobenzaldehyde reagent (PDB) according to Dent (6).

The R_f -values of urea and citrulline are

	n-butanol	phenol-NH ₃
urea	0.29	0.72
citrulline	0.02	0.54

They thus differ distinctly from each other (cf. Figure 12). An intensive spot of citrulline was obtained from all extracts examined but a detectable amount of urea was present only in the extract of unnodulated roots (about 0.005 per cent of dry weight). The presence in the extracts of ureides other than citrulline could not be proved with certainty. It was found that for some experiments with the root and nodule extracts of *A. glutinosa* two yellow very faint extra spots were obtained in addition, but their identification is still uncertain since some amino acids also give a faintly positive reaction (see experimental part).

Photometric determination of citrulline

For control a photometric determination of citrulline was also made from prep. no. 5 according to Kawerau (9) with diacetyl monoxime reagent, which reacts with urea and ureides. Because of its intense brown colour the extract had to be decolorized by treatment with active carbon whereby about 10 per cent of the total nitrogen of the extract was absorbed in the carbon.

The yield obtained was 151 μg citrulline/100 mg dry wt. On this basis with the presumption that approximately 10 per cent of the citrulline was also absorbed by the carbon, we obtained for the citrulline content the value of about 166 μg /100 mg dry wt., which agreed well with the paperchromatographic determinations (170 μg). Since the preparations had to be decolorized with carbon whereby a difficultly controllable absorption of citrulline took place, this method is not much more accurate than the paper chromatographic reference series technique. In addition the latter is much more reliable if urea is present. Therefore, photometric determinations were not made from other samples.

Experimental

Preparation of 70 per cent ethanol extract

N g. of fresh plant material (containing ca. 75 per cent water), of which a representative sample was taken for dry wt. determination (to constant weight at 105° C), were crushed in 2.2 N ml. of absolute ethanol, allowed to stand overnight and centrifuged. A 70 per cent by wt. alcohol solution was obtained. The precipitate was extracted twice with at least a double volume of 70 per cent by wt. alcohol. The extracts were combined, evaporated in vacuo to dryness and the precipitate was dissolved in a known volume with water. To prevent infection a drop of toluene was added to the solution. By this extraction method about 96—97 per cent of the ethanol-soluble nitrogen was extracted.

Determination of total nitrogen

Determinations were made by microkjeldahl from samples dried in a ventilated oven at ca. 80° C.

Determination of ammonia nitrogen

Ammonia was determined according to Pucher et al. (14) using fresh samples of leaves and nodules, care being taken to avoid splitting ammonia from labile compounds, like amides. Samples were handled using various techniques.

a) Leaves were weighed and cut into narrow strips, which were rapidly crushed in a mortar and washed quantitatively into the destillation flask. Borate buffer was added and the destillation made.

Result $1.25 \pm 0.10 \mu\text{g NH}_3\text{-N}/100 \text{ mg dry wt.}$

b) As in paragraph a, but the crushed sample was boiled for 1 minute and cooled again before adding the borate buffer. (The boiling was to effect the complete lysis of the cells and inactivation of the enzymes.)

Result $2.4 \pm 0.10 \mu\text{g NH}_3\text{-N}/100 \text{ mg dry wt.}$

c) The cell sap was pressed from fresh leaves with a hydraulic press. This manipulation took several hours. Before the determinations were made, the sap stood in an ice box for 3 days.

Result $1.35 \pm 0.10 \mu\text{g NH}_3\text{-N}/100 \text{ mg dry wt.}$

The results obtained from methods a and c were comparable and have been supposed to be nearest to the correct value.

Ammonia determinations from the nodules were made according to a and c. The results were:

a) Immediately after crushing: $3.90 \pm 0.10 \mu\text{g NH}_3\text{-N}/100 \text{ mg dry wt.}$

c) From press sap after standing in an ice box for 3 days: $30.5 \mu\text{g NH}_3\text{-N}/100 \text{ mg dry wt.}$

The press sap of the nodules evidently contains enzymes which are capable of rapidly freeing ammonia. It is possible that for this same reason the value obtained in paragraph a) may also be too high.

Purification of amino acids by ion-exchange resin

Purification of amino acids was made with Dowex-2 anion exchange resin according to the principle of Davies, Hughes and Partridge (5). In the manner described above an amino acid extract was prepared from 136.5 g of alder nodules harvested in October 1951 (dry wt. 37.6 g). The dark brown extract obtained amounted to 100 ml. 36 ml of Dowex-2 ion exchange resin was thoroughly washed with 4 N HCl, water, then with 2 N NaOH and finally with distilled CO₂-free water, until a neutral reaction was obtained, whereupon the resin was packed tightly to a 15 cm high column. 93 ml of this extract corresponding to 35 g dry weight, were passed into the column by nitrogen under pressure after which CO₂-free water was allowed to run through the column until it came out colourless (abt. 100 ml). Only a small fraction of the pigments was absorbed in the column the main part passing through immediately. It was ascertained by paper chromatography that practically all amino acids were absorbed.

Amino acids were displaced from the column using 1 per cent acetic acid and the eluate collected in 10 ml fractions by an automatic fraction collector. The fractions were examined paper chromatographically, and it was noted that practically all amino acids had come through in fractions 23—30 (230—300 ml). These fractions were combined and concentrated to 50 ml. Paper chromatographic analysis revealed that their amino acid composition was qualitatively the same as before treatment with resin.

Isolation of L(+)-citrulline as Cu-salt

50 ml of the amino acid extract purified with ion exchange resin was treated with 1 g CuO by boiling under a reflux condenser for $1/2$ h and filtered off from the CuO which had not reacted.

On standing overnight 159 mg of light-blue Cu-salt of silk lustre was crystallized from the filtrate, m.p. 227—233° (N=19.40 per cent). When recrystallized from water it melted on rapid heating foaming at 266—268° C, on slow heating it carbonized at 252.5° C without melting. N=20.1 per cent (Kjeldahl). $(C_6H_{12}N_3O_3)_2Cu$ contains in theory 20.4 per cent N.

The authentic Cu-L(+)-citrulline also melted at 266—268° C, as did the mixture of both salts. (It should be mentioned that the melting point of Cu-DL-citrulline is 257—258° C. It also differs in colour from the optically active salt by being violet blue).

When the mother liquor was concentrated to about 2 ml and allowed to stand for 2 days in an ice-box, a further 50 mg of Cu-L(+)-citrullinate (m.p. 240° C) was crystallized, hence, the total yield was 209 mg raw product from 35 g dry weight.

Paperchromatographic technique

Amino acids

The paper chromatograms were prepared in the manner illustrated in Figure 12. With this solvent combination satisfactory chromatograms can be obtained from the concentrated ethanol extracts without further purification. With butanol acetic acid contra phenol or mesityl-oxide formic acid contra phenol this is not the case. Plant extracts tend to give irregular chromatograms with anomalous R_f -values and shape of spots.

The chromatogram of Figure 12 contains inter alia all the other protein amino acids except leucine, iso-leucine, phenylalanine and tryptophane, which were allowed to pass the bottom edge of the paper in order to effect a better separation of slower-moving amino acids. To ascertain the absence of the above mentioned amino acids one-dimensional chromatograms were prepared from all extracts.

The colour of the amino acid spots was obtained by spraying with 0.2 per cent ninhydrin solution in water-saturated butanol, with which all compounds in Figure 12, except urea, reacted.

With reference to the amino acid map in Figure 12 it should be pointed out that glutathione (Hoffman-La Roche) has regularly given two spots, 19 A and B, under conditions of our experiments. Lindan and Work (11) have studied in detail the paper chromatography of glutathione. They obtained three spots with phenol-collidine run. The dependence of the R_f -value of γ -amino butyric acid on pH has been described above. Synge (19) has also noticed that the R_f -value of β -alanine as a function of pH changes more than that of α -amino acids. However, under the conditions of our experiments it has always travelled at the same speed with respect to α -amino acids; hence, small differences in the pH of the sample did not affect its travelling velocity. The R_f -value of ethanolamine is very sensitive to the pH. This spot can be located in 4 different places, 45 A—D, and even between them. In both solvents the slower spot corresponds to more acid conditions. The shape of the spots is often irregular and more than one of them may occur simultaneously.

As is already known (Arnoff, 1), alkaline amino acids may also give several spots. In certain cases we have obtained two spots even from acid amino acids. Figure 4 shows a chromatogram in which glutamic acid has in part proceeded as a rapid form 17 B. This, however, does not happen except under very acid conditions.

In all uncertain cases the identity of amino acids was confirmed by running mixture chromatograms with an authentic preparation. The acid atmosphere in the chromatographic runs was accomplished by placing 5 drops of conc. HCl in a bowl at the bottom of the chamber, the alkaline atmosphere by 100 ml. of 0.5 per cent NH_3 . In all runs HCN was used to prevent the pink front. For chambers we have used all-glass aquarium tanks 65 cm long, 60 cm high and 48 cm broad which hold 16 whole sheets of Whatman No. 1 paper in 4 troughs, 4 in each, an arrangement suggested by Lindan and Work (11).

Detection of urea and ureides

For the development of colour 5 per cent p-dimethyl aminobenzaldehyde was used in approx. 90 per cent ethanol, which was 3 N with respect to hydrochloric acid. This reagent reacts with urea and ureides forming yellow pigments almost immediately, even at room temperature. Many amino acids, especially glutamic acid and arginine, also give a yellow spot with PDB, which, however, appears very slowly, requiring several hours at room temperature and not attaining full intensity until after some days. Indole and its derivatives, e.g. indolyl acetic acid and tryptophane, produce a slowly-developing violet spot.



Fig.13.

Photometric determination of citrulline

The method of Kawerau (9) was followed in slightly modified form: A ml (0—2 ml) of sample + 2 ml of diacetyl reagent (1000 mg diacetylmonoxime + 5 ml glacial acetic acid made up with water to 100 ml) + 2 ml acid mixture (10 g arsenic acid made up to 100 ml with 36 per cent HCl) + 2-A ml water were pipetted into an accurately calibrated flask of 6.00 ml¹ (Figure 13) which was kept for 15 min. at constant temp. of 100° C in a water-bath. It was then cooled for 2 min in cold water, the condensation water dried off from the upper part of the tube down to the mark, made up to the volume with water, and stirred. The extinction was measured with a Beckmann spectrophotometer at 500 m μ . The reference line was constructed with known amounts of citrulline.

Determinations were made in series of 12, of which 4 were control determinations with known amounts of citrulline (0—150 μ g). In this way, a standard line was obtained for each series. This improved the accuracy, for the extinction values corresponding to the same amounts of citrulline may vary even 2—3 per cent in different determinations owing to uncontrollable factors.

Discussion

The abundance of citrulline in the roots and nodules of alder and the complete absence of asparagine and glutamine are the most striking differences in the composition of soluble N of alder and the leguminous plants. The case is completely the reverse in the roots and nodules of legumes,

¹ Easily made from a 14 mm Pyrex test tube and calibrated to 6 ml by mercury.

glutamine and especially asparagine being very abundant, but citrulline being lacking (Hunt, 8, Virtanen et al., 22).

The alder leaves contain much less citrulline than the roots and nodules of the same plant, although the content of other amino acids is in the leaves even higher (on dry weight basis).

The amino acid metabolisms in the roots and leaves of alder seem thus to differ from each other. The great abundance of citrulline is characteristic of the alder roots, but does not, however, originate specifically from the symbiosis with nodule bacteria because it is found even in entirely un-nodulated roots, though in much smaller quantities.

The abundance of γ -amino butyric acid together with citrulline in the nodules of *A. incana* deserves to be mentioned. It has been shown in recent years that this amino acid in higher plants forms an important component of soluble nitrogen (see the literature references in the paper by Synge, 19). It is evident, that in most cases γ -amino butyric acid is formed from glutamic acid by decarboxylation, since several workers have found L-glutamic acid decarboxylases in higher plants (Okunuki, 12, Schales and Schales, 16, Hasse and Schumacher, 7). However, Steward and Thompson (13) have observed potato tubers to be rich in γ -amino butyric acid but found only minimal glutamic acid decarboxylase activity in them, suggesting, therefore, that γ -amino butyric acid may be the precursor of glutamic acid by carboxylation rather than a decarboxylation product. Hasse and Schumacher (7) have also found low L-glutamic acid decarboxylase activity in potato. As will be shown in a separate publication, we have found L-glutamic acid decarboxylase activity in the leaves and nodules of *A. incana* (23). This activity seems to be located in the insoluble cell particles. It is therefore probable, that γ -amino butyric acid is formed in alder from L-glutamic acid by decarboxylation.

As far as we know citrulline has hitherto not been found in higher plants except in water-melon (*Citrullus vulgaris*) from which it was originally isolated by Wada (21) as racemate in 1930. It is very likely that it is synthesized in the roots of alder from ornithine via the Krebs-Henseleit urea cycle (10) as in the animal organism. This is suggested by the remarkably high concentration of ornithine and arginine in the alder roots in winter. The occurrence of Krebs-Henseleit cycle in the alder has, however, not yet been convincingly proved.

As far as we know, the operation of this cycle has not yet been shown in the higher plants, but Srb and Horowitz (17) have genetically proved its existence in the mould *Neurospora crassa*.

Among the results described above one of the most interesting is the noticeably higher concentration of free amino acids in those parts of the

roots which are nearer to the nodules, when compared with the more remote parts. The nodules seem to form a nitrogen store in winter and the metabolic activity seems to be at the highest in them or in those parts of the roots nearest to them. Only traces of arginine and ornithine are found in the nodules but there are large quantities in those parts of the roots nearest to the nodules.

The enzymatic mechanism of the urea cycle in the animal organism has been much studied in recent years and some of its details have been elucidated.

The synthesis of citrulline from ornithine includes several reactions. The ureido group of citrulline is evidently first built up on the amino group of glutamic acid and then transferred to the δ -position of ornithine as a unity by a »group transfer reaction» (Cohen and Grisolia, 3). The actual intermediate is not known but is evidently related to α -ureido-glutamate.

The imination of citrulline to arginine is effected specifically by aspartic acid, as has been shown by Ratner and Pappas (15) with a purified enzyme preparation. The fact that the reaction proceeds in tissues more rapidly with glutamic acid as the amino donor than with aspartic acid, as was shown by Borsook and Dubnoff in 1941 (2), is explained by the fact that glutamic acid is not only able to produce aspartic acid by transamination, but is more easily oxidized in the tissue than aspartic acid, thus also being able to produce the energy necessary for the reaction.

For the present it is difficult to advance any hypothesis for the possible role of the assumed ornithine cycle in the roots and nodules of alder except that of an easily controlled mechanism for storing and transporting nitrogen. As will be shown in a separate publication, the alder is completely devoid of arginase activity. Therefore the citrulline nitrogen cannot be mobilized in the alder through urea. Urea is in fact not found in the alder tissue except in negligible amount. The great accumulation of citrulline in the root system of alder in autumn obviously makes possible a rapid synthesis of arginine in spring and, through glutamic acid, of other amino acids. Furthermore, it is possible that citrulline has other roles in the nitrogen metabolism which are not yet known.

Summary

1) Paper chromatographic analysis revealed that citrulline is the pre-dominating free amino acid in the root nodules of *A. incana* and *A. glutinosa* throughout the year. In autumn its concentration has increased 5—10 fold, compared with the summer value.

2) The nodules of *A. incana* contain in addition to citrulline much γ -amino butyric acid, which evidently is formed from L-glutamic acid, since L-glutamic

acid decarboxylase activity was found to be present in the insoluble residue of the crushed nodules.

3) The roots of *A. glutinosa* contain in addition to citrulline much arginine and ornithine but the nodules have only traces of them. The concentration of free amino acids in the roots near to the nodules is about 5 times that in the more remote parts if calculated per unit dry matter.

4) The completely unnodulated roots of uninoculated alders grown on combined nitrogen also contain citrulline, hence, it is not a compound specific to nitrogen fixation.

5) The stems and especially the leaves of alder contain considerably less citrulline and soluble nitrogen in general than the roots of the same plant.

6) The presence of urea and the identity of citrulline were tested paper chromatographically using p-dimethylaminobenzaldehyde reagent. Only in unnodulated roots was urea found and then only in very small quantities (about 0.005 per cent of dry wt.), possibly as the result of a secondary splitting. The identity of citrulline in all extracts was confirmed.

7) The arginase activity of the alder nodules, roots and leaves was tested. No activity could be detected.

8) The amino acids extracted with ethanol were separated from many impurities in the extract by means of alkaline ion exchange resin Dowex 2.

9) L(+)-citrulline was also isolated from the nodules of *A. incana* as the Cu-salt. This was identified by means of the mixed melting point with the authentic Cu-L(+)-citrullinate.

10) In the nodule extract of *A. incana* two unknown compounds were found, possibly peptides, which disappear in the HCl-hydrolysis. Simultaneously alanine, glutamic acid, some glycine and possibly γ -amino butyric acid are set free.

11) Citrulline was noted to break down to a great extent to ornithine in the HCl-hydrolysis.

12) In one preparation citrulline was also determined photometrically by diacetylmonoxime reaction.

13) Hypotheses are advanced on the significance of the observations.

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References

1. Aronoff, S.: Separation of the ionic species of lysine by means of partition chromatography. — *Science* 110: 590. 1949.
2. Borsook, H. & Dubnoff, J. W.: The conversion of citrulline to arginine in kidney. — *J. Biol. Chem.* 141: 717. 1941.

3. Cohen, P. P. & Grisolia, S.: The rôle of carbamyl-L-glutamic acid in the enzymatic synthesis of citrulline from ornithine. — *J. Biol. Chem.* 182: 747. 1950.
4. Crumpler, H. R. & Dent, C. E.: Distinctive test for α -amino acids in paper chromatography. — *Nature (London)* 164: 441. 1949.
5. Davies, C. W., Hughes, R. B. & Partridge, S. M.: Amino acid separations by use of a strong-base resin. — *J. Chem. Soc. (London)* 1950, p. 2285.
6. Dent, C. E.: A study of the behaviour of some sixty amino-acids, and other ninhydrin-reacting substances on phenol-collidine filter-paper chromatograms, with notes to the occurrence of some of them in biological fluids. — *Biochem. J.* 43: 169. 1948.
7. Hasse, K. & Schumacher, H.-W.: Das Reaktionsprodukt der Decarboxylierung von L-Glutaminsäure mittels pflanzlicher Decarboxylase. — *Chem. Ber.* 83: 68. 1950.
8. Hunt, G. E.: A comparative chromatographic survey of the amino acids in five species of legume roots and nodules. — *Am. J. Botany* 38: 452. 1951.
9. Kawerau, E. — *Sci. Proc. Dublin Soc.* 24: 63. 1946. (Cf. *C. A.* 40: 50857. 1946).
10. Krebs, H. A. & Henseleit, K.: Untersuchungen über die Harnstoffbildung im Tierkörper. — *Z. physiol. Chem.* 210: 33. 1932.
11. Lindan, O. & Work, Elizabeth: The amino-acid composition of two yeasts used to produce massive dietetic liver necrosis in rats. — *Biochem. J.* 48: 337. 1951.
12. Okunuki, K. — *Botan. Mag. (Tokyo)* 51: 270. 1937. (cf. ref. 7 in this paper by Hasse et al.).
13. Polson, A.: Quantitative partition chromatography and the composition of *E. coli*. — *Biochim. et Biophys. Acta* 2: 575. 1948.
14. Pucher, G. W., Vickery, H. B., & Leavenworth, C. S.: Determination of ammonia and of amide nitrogen in plant tissue. — *Ind. Eng. Chem., Anal. Ed.*, 7: 152. 1935.
15. Ratner, S. & Pappas, A.: Biosynthesis of urea. I. Enzymatic mechanism of arginine synthesis from citrulline. — *J. Biol. Chem.* 179: 1183. 1949. II. Arginine synthesis from citrulline in liver homogenates. — *Ibid.* 179: 1199. 1949.
16. Schales, O., Nims, V. & Schales, S. S.: Glutamic acid decarboxylase of higher plants. I. Distribution; Preparation of clear solutions; Nature of prosthetic group. — *Arch. Bioch.* 10: 455. 1946.
17. Srb, A. M. & Horowitz, N. H.: The ornithine cycle in *Neurospora* and its genetic control. — *J. Biol. Chem.* 154: 129. 1944.
18. Steward, F. C. & Thompson, J. F.: The nitrogenous constituents of plants with special reference to chromatographic methods. — *Ann. Rev. Plant Phys.* 1: 251. 1950.
19. Syngé, R. L. M.: Methods for isolating ω -amino acids: γ -amino butyric acid from rye grass. — *Biochem. J.* 48: 429. 1951.
20. Wada, M.: Isolierung des Citrullins (δ -Carbamido-ornithin) aus tryptischen Verdauungsprodukten des Caseins. — *Biochem. Z.* 257: 1. 1933.
21. — Über Citrullin, eine neue Aminosäure im Presssaft der Wassermelone, *Citrullus vulgaris* schrad. — *Biochem. Z.* 224: 420. 1930.
22. Virtanen, A. I., Miettinen, J. K., Moisio, T., & Kari, S., 1951. — Unpublished.
23. Virtanen, A. I. & Miettinen, J. K. 1952. — Unpublished.
24. Virtanen, A. I. & Saastamonen, S.: Untersuchungen über die Stickstoffbindung bei der Erle. — *Biochem. Z.* 284: 72. 1936.
25. Virtanen, A. I. & Saubert-von Hausen, S. 1951. — Unpublished.

The Assimilation of Ammonia by Nitrogen-Starved Cells of *Chlorella vulgaris*. III. The Effect of the Addition of Glucose on the Products of Assimilation.

By

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Introduction

Syrett (1953) found that nitrogen-starved cells of *Chlorella vulgaris* assimilated ammonia-N very rapidly. After 3 hrs. most of the nitrogen assimilated was present in the cells as soluble organic nitrogen. This paper shows what happened to the assimilated nitrogen when longer experiments were performed.

Experimental

Material. The strain of *Chlorella vulgaris* used was that studied previously (Fowden 1951 a, Syrett 1951, 1953). The cells were grown as pure cultures in the medium used by Syrett (1953), in which nitrogen was supplied as ammonium nitrate. When a nitrogen-free medium was required the ammonium nitrate was omitted. The media were buffered with $\frac{1}{15}$ M phosphate, at pH 6.0—6.1. The cultures were grown at 25° and illuminated by a tungsten filament lamp giving a light intensity of 600 f.c. at the culture surface. The cultures were aerated by a stream of sterile air containing 0.5 per cent CO₂, flowing at the rate of 10 l./hr. through each culture vessel. Further details of growth conditions are given by Syrett (1951).

In order to obtain nitrogen-starved cells, normal cells which had been growing in the complete medium for 74—78 hrs. were harvested by centrifuging at 360 g. for 5 min. The cells were washed twice with and resuspended

in sterile nitrogen-free medium, and then maintained for a further 16—20 hr. under the growth conditions described above but with an increased gas flow of 20 l./hr. per culture. Immediately before starting the ammonia assimilation experiments, the cells were centrifuged, washed once and resuspended in sterile nitrogen-free medium to give a final concentration of about 8 mg. dry wt. cells per ml.

Assimilation of ammonia. 150 ml. of this cell suspension, in a 500 ml. conical flask was shaken at the rate of 120 strokes/min. in a darkened Warburg tank at 25°. A 10 ml. sample was removed and used to determine the initial intracellular nitrogen distribution. Ammonium sulphate solution was then added to the remainder of the suspension to give a final ammonium ion concentration of 10 μ mol/ml. Further samples of 10 ml. were withdrawn at intervals over a period of 24 hrs. Each sample was centrifuged at 800 g. for 5 min. The supernatant liquid was decanted and saved for ammonia estimations, while the tube containing the cells was heated for 2 min. at 100°. The soluble nitrogenous constituents were extracted from the cells using successive 8, 5 and 5 ml. portions of distilled water at 80° for 10—15 min., cell debris being removed by centrifuging after each extraction. The combined extracts were finally made up to 25 ml. and stored under toluene at 3°.

Four 2 ml. samples were also removed during the course of the experiment; two were used for dry weight and two for total nitrogen determinations.

Although the medium and flasks were sterile at the beginning of the experiment, no precautions were taken to prevent contamination during sampling. No contamination was observed other than in the 24 hrs. sample. Little importance was attached to the determinations made on this last sample.

A second experiment was performed in which glucose was added to the nitrogen-starved cells 15 min. before the addition of ammonium sulphate to give an initial glucose concentration of 1 per cent.

Samples of 'normal' cells grown for 3 days on the complete medium were also extracted in the same way.

Analytical methods. Unless otherwise stated these were the same as those used by Syrett (1953). Duplicate determinations, which generally agreed to within 3 per cent, were always made.

The following nitrogen fractions were determined on the cell extracts: — (a) ammonia-N, (b) amide-N, (c) free α -amino-N, (d) combined α -amino-N (released by hydrolysis with 6 N \cdot HCl at 100° for 24 hrs.), (e) total soluble-N, and (f) the α -amino-N of the basic amino-acids. We used the method of Moore and Stein (1948) for estimations of α -amino-N after the removal of ammonia. The α -amino-N of the basic amino-acids was determined using

the quantitative paper chromatographic method of Fowden (1951 b). After removing any glutamine or asparagine in the extracts by hydrolysis with $N \cdot HCl$ at 100° for 3 hrs., the basic amino-acids were separated as a composite spot from the remaining amino-acids during a 24 hrs. development on Whatman no. 4 paper using the *n*-butanol-acetic acid mixture of Partridge (1948) as solvent. A solution containing equimolar amounts of arginine, histidine and lysine was used as a colorimetric standard for these determinations. Estimations were in triplicate; the deviations of single results from the mean never exceeded 10 per cent of the latter figure.

The insoluble nitrogen of the cells was calculated from the total nitrogen figures by subtracting the estimated values for soluble nitrogen.

Results

The results of the first experiment are shown in fig. 1.

Prolonged development of chromatograms of the cell extracts showed that the basic amino-acids present were arginine, histidine, ornithine and lysine. Arginine appeared to be present in quantities approximately equal to those of lysine and ornithine together, while less histidine was present. Since arginine contains four nitrogen atoms, histidine three, and lysine and ornithine two each, the average nitrogen content of the basic amino-acids present in the extracts was roughly 3 atoms per molecule. The values of the nitrogen present in the basic amino-acids plotted in the figures have been calculated by multiplying the determined α -amino-N values by three. When this was done all the soluble organic nitrogen could be accounted for as amide-N or as free or combined amino-acid nitrogen except that the correction appeared to overestimate the nitrogen present in the basic amino-acids in the first 14 min. of experiment 1 (Table 1.).

Without this correction the determined α -amino-N and amide-N account for only 55 to 85 per cent of the total soluble nitrogen (see also Syrett 1953).

After the exhaustion of the added ammonia, the cells still contained a higher proportion of their total nitrogen as soluble organic nitrogen than that found in 'normal' or 'nitrogen-starved' cells; in particular the basic amino-acid nitrogen was high (see Table 2). It appeared possible that the cells contained enough carbohydrate reserves to assimilate all the added ammonia but insufficient to complete the assimilation into protein and other insoluble compounds. In order to test this possibility the second experiment was performed in which glucose was added; the results are shown in Fig. 2 and Tables 1 and 2.

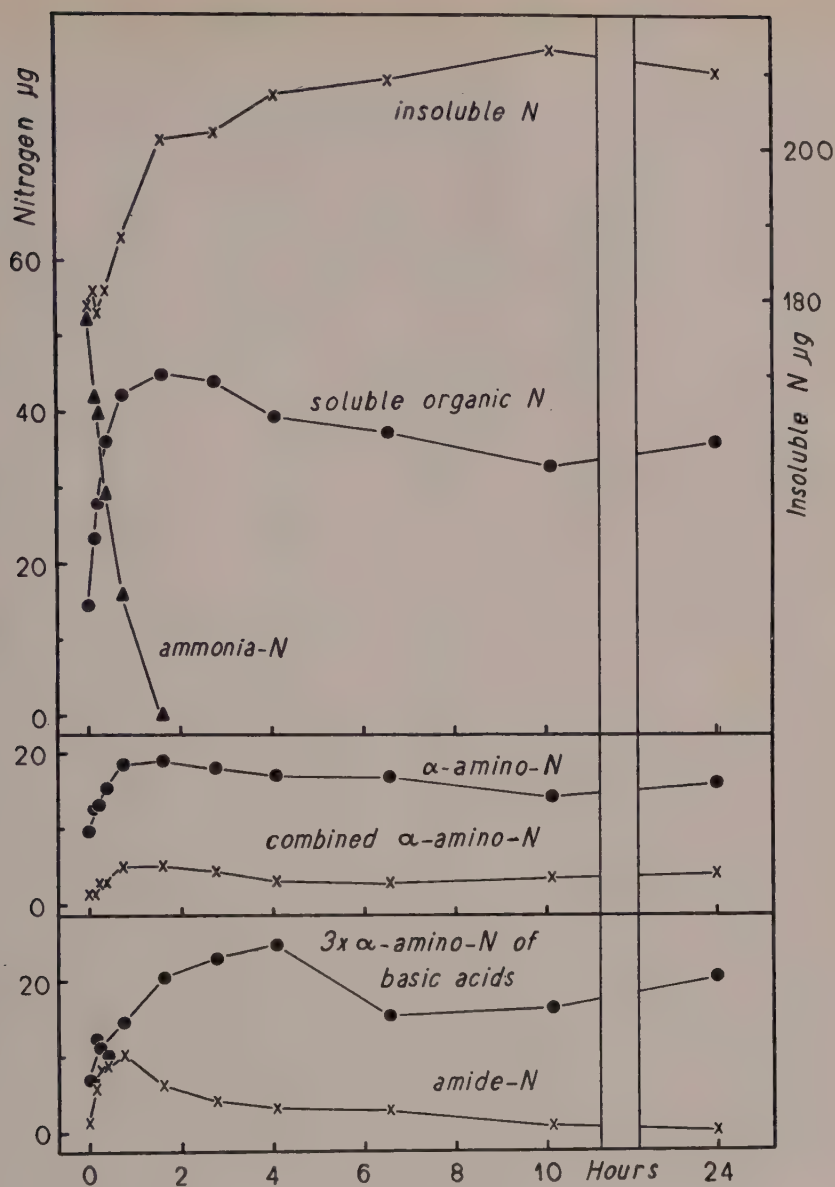


Fig. 1. Experiment 1 — No glucose added.

10 micromoles ammonia were added to each ml. of cell suspension at zero time. The data graphed show the quantities of the various nitrogen fractions present in 0.4 ml. suspension. (This volume of suspension, after extraction, yielded 1 ml. extract.)

Dry wt. of suspension = 9.3 mg./ml.

∴ Dry wt. of cells which yielded 1 ml. extract = 3.72 mg.

Table 1. Comparison of the sum of the determined nitrogen fractions with the total soluble organic nitrogen of the extracts.

Expt.	Sampling Time. (Mins)	Percentage of Soluble Organic-N accounted for by Amide-N+Free and Combined α -amino-N	Percentage of soluble Organic-N accounted for by Amide-N+Free and Combined α -Amino-N + 2 \times α -amino-N of basic amino-acids	Residual Free α -amino-N i.e. Total Free α -amino-N - α -amino-N of Amides and α -Amino-N of basic amino-acids (μ g Nitrogen per ml extract)	Amide-N (μ g Nitrogen per ml extract)	α -Amino-N of basic acids as % of total free α -Amino-N
Expt. 1	0	84.3	116.0	6.0	1.3	24
	9	84.0	119.2	2.8	5.7	33
	14	86.7	113.2	1.2	8.3	28
	24	75.5	94.0	3.4	8.7	22
	45	80.0	102.8	3.5	10.2	26
	97	67.5	97.5	6.0	6.2	36
	166	60.4	95.0	6.2	4.2	42
	245	59.0	100.5	5.6	3.2	48
	394	60.0	87.1	8.8	2.9	31
	608	56.0	88.8	8.0	0.8	38
	1474	55.2	92.5	9.1	0	43
	No added glucose					
Expt. 2	0	72.0	100.8	4.1	0	31
	11	85.4	112.7	5.1	2.9	26
	23	72.0	90.4	5.3	5.3	23
	41	77.5	101.2	3.8	7.7	28
	93	66.5	81.4	4.7	8.3	21
	161	64.5	84.0	6.3	3.5	26
	260	68.5	99.6	4.6	1.6	38
	393	61.9	94.9	3.6	0.8	42
	544	65.2	96.6	3.3	0.6	45
	1431	92.8	146.2	3.3	0.2	42
	1 % glucose added					

Ammonia was again rapidly assimilated to soluble organic nitrogen but after 10 hrs. there was only slightly more soluble organic nitrogen present than in the initial 'nitrogen-starved' cells. The percentage of nitrogen present in the basic amino-acids was still higher than initially.

Discussion

In both experiments (see figs. 1 and 2 and Table 1) ammonia was rapidly assimilated to soluble organic nitrogen. In the first 20 min. after the addition of ammonia appreciable amounts of amides and free amino-acids were formed, whilst the insoluble nitrogen remained fairly constant. There was also a smaller increase in the combined α -amino-N, although this was not

Table 2. A comparison of the nitrogen fractions of normal cells with those of the nitrogen-starved cells used for Experiments 1 and 2.

Figures given are $\mu\text{g.}$ -nitrogen per mg. dry wt. cells.

The figures in parentheses represent the nitrogen of the fraction as a percentage of the total cell nitrogen.

Cells	Soluble-N	Free α -Amino-N	$3 \times$ α -Amino-N of basic acids	Combined α -Amino-N	Insoluble-N	α -amino-N of basic acids as % of total free α -amino-N
Normal	6.5 (7.8)	3.9 (4.7)	2.4 (3.0)	0.7 (0.9)	75.1 (92.0)	21
Nitrogen-starved. Initial	3.9 (7.5)	2.6 (4.9)	1.9 (3.5)	0.4 (0.7)	48.1 (92.3)	24
Expt. 1. No added glucose. 10 hrs after addi- tion of Ammonia	8.9 (13.4)	3.8 (5.8)	4.4 (6.6)	0.9 (1.4)	57.3 (86.6)	38
Nitrogen-starved. Initial	4.7 (6.4)	2.1 (2.9)	2.0 (2.7)	1.3 (1.7)	69.2 (93.7)	31
Expt. 2. 1 % glucose added. 10 hrs after addi- tion of Ammonia	5.9 (6.4)	2.5 (2.7)	3.3 (3.6)	1.2 (1.3)	86.2 (93.5)	45

as large as that reported by Syrett (1952), when hot 0.1 per cent trichloroacetic acid was used for extraction instead of the hot water used in the present experiments. The trichloroacetic acid may have extracted some combined amino-N which was insoluble in hot water alone.

The rapid increase of amide-nitrogen was presumably due mainly to the synthesis of glutamine (see Syrett 1953). Table 1 contains values for the 'residual' α -amino-N calculated by subtracting the α -amino-N present in the amides and basic amino-acids from the total free α -amino-N. In experiment 1 the increase in amide-nitrogen in the first 14 min. was accompanied by a fall in the residual α -amino-N. Thus it appears that some of the amide was synthesised from amino-acids already present in the cells. In experiment 2 where glucose was present, there was no marked change in the residual α -amino-N and amino-acids must have been synthesised as quickly as they were converted to amides.

Rapid amide synthesis following ammonia feeding has been reported by Street (1949) and Roine (1947), and the suggestion (Christiansen and Thi-mann, 1950) that their formation is closely connected with protein synthesis was supported by the present experiments, in which large increases of insoluble nitrogen occurred after 20 min.

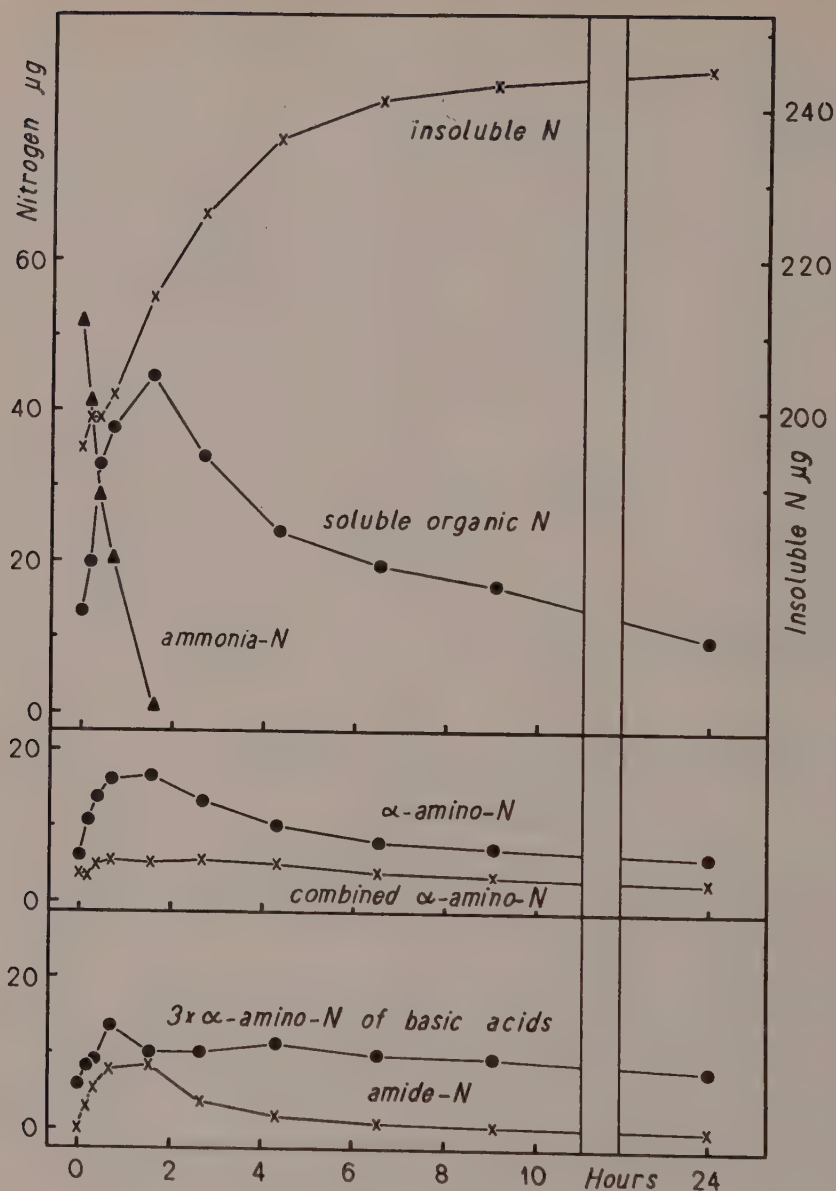


Fig. 2. Experiment 2 — Glucose added giving a final concentration of 1 per cent.

10 micromoles ammonia were added to each ml. of cell suspension at zero time. The data graphed show the quantities of the various nitrogen fractions present in 0.4 ml. suspension. (This volume of suspension, after extraction, yielded 1 ml. extract.)

Dry wt. of suspension = 7.05 mg./ml.

\therefore Dry wt. of cells which yielded 1 ml. extract = 2.82 mg.

The amounts of the basic amino-acids formed differed appreciably in the two experiments. When glucose was present (expt. 2) they were formed rapidly after the addition of ammonia but soon reached a constant level (fig. 2), although they continued to form an increasing percentage of the free α -amino-N (Table 1), due to the disappearance of other amino-acids. In the absence of glucose, the basic amino-acids increased much more (fig. 1), and continued to increase even when other soluble nitrogenous fractions were decreasing. Fig. 1 suggests that the amide-nitrogen which disappeared after the first 45 min. may have been incorporated into the basic amino-acids. The percentages of the free α -amino-N contributed by the basic amino-acids in both experiments were much higher than the corresponding figure of about 12 per cent found for a *Chlorella* protein hydrolysate by Fowden (1951 a).

In the absence of glucose a large proportion of the assimilated nitrogen remained in the cells in the form of soluble organic compounds, and the rate of conversion of soluble nitrogen into protein nitrogen was quite slow after the first 2 hrs. When glucose was present, the soluble organic nitrogen reached a maximum value after 90 min., and then fell eventually to its original 'nitrogen-starved' level, whilst active protein synthesis continued for some 6 hrs. It would appear that the cells contained insufficient carbon reserves in the absence of glucose (expt. 1) to complete the assimilation of the added nitrogen to protein, and that at the end of the experiment they contained an excess of nitrogen, a large proportion of which was present as soluble basic amino-acids.

Summary

(1) The uptake of ammonia by 'nitrogen-starved' cells of *Chlorella vulgaris* was studied. Rapid assimilation of $\text{NH}_3\text{-N}$ into soluble organic nitrogenous compounds, principally amide and free amino-acid nitrogen, occurred.

(2) In the presence of glucose, the soluble organic nitrogen finally returned to the original nitrogen-starved level, the assimilated $\text{NH}_3\text{-N}$ being incorporated into insoluble (protein) nitrogen. When no glucose was added, a large proportion of the assimilated nitrogen remained in the form of soluble organic compounds.

(3) In both cases basic amino-acids represented a high percentage of the free amino-acids formed during ammonia assimilation, and even after 24 hrs. this percentage remained at a higher level than that found initially or in 'normal' cells.

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References

- Christiansen, G. S. & Thimann, K. V.: The metabolism of the stem tissue during growth and its inhibition. III. Nitrogen metabolism. — Arch. Biochem. 28: 117. 1950.
- Fowden, L.: The composition of the bulk protein of *Chlorella*. — Biochem. J. 50: 355. 1951 a.
- The quantitative recovery and colorimetric estimation of amino-acids separated by paper chromatography. — Biochem. J. 48: 327. 1951 b.
- Moore, S. & Stein, W. H.: Photometric ninhydrin method for use in the chromatography of amino-acids. — J. Biol. Chem. 176: 367. 1948.
- Partridge, S. M.: Filter-paper partition of sugars. — Biochem. J. 42: 238. 1948.
- Roine, P.: On the formation of primary amino-acids in the protein synthesis in yeast. — Ann. Acad. Sci. Fennicae. Ser. A. II. Chem. No. 26. I. 1947.
- Street, H. E.: Nitrogen metabolism of higher plants. — Advanc. Enzymol. 9: 391. 1949.
- Syrett, P. J.: The effect of cyanide on the respiration and the oxidative assimilation of glucose by *Chlorella vulgaris*. — Ann. Bot. 15. (N.S.): 473. 1951.
- Ann. Bot. (in press). 1953.

On the Growth-Regulating Effects of Some 1-Naphthyl and 2,4-Dichlorophenoxy Derivatives without Carboxyl Groups

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Introduction

It is generally assumed that one of the structural requirements for high auxin activity of a molecule is the presence of a carboxyl group in the side chain (14). In the search for auxin antagonists it was therefore close at hand to test substances with »active» nuclei but with a side chain devoid of such a group. 2,4-Dichloroanisole was thus tested by Bonner (6) and 1-naphthole by Åberg (1). Much more effective antagonists were soon discovered (1—4, 7—9, 13), however, and, as these were all carboxylic acids, it became pertinent to study in how far the carboxyl group was a necessary requirement for strong antagonistic activity also. To this purpose the following substances were used:

Ne: methyl 1-naphthylmethyl ether, $C_{10}H_7 \cdot CH_2 \cdot O \cdot CH_3$, (PAL Chemicals Ltd., London).

Nm: 1-naphthyl-nitromethane, $C_{10}H_7 \cdot CH_2 \cdot NO_2$, (PAL).

NAn: 1-naphthyl-acetonitrile, $C_{10}H_7 \cdot CH_2 \cdot CN$, (Eastman Kodak Co., Rochester).

NAd: 1-naphthyl-acetamide, $C_{10}H_7 \cdot CH_2 \cdot CO \cdot NH_2$, (Eastman Kodak).

Nf: 1-naphthole, $C_{10}H_7 \cdot OH$, (Merck, pro analysi) (see publ. 1).

2,4-Da: 2,4-dichloroanisole, $C_6H_3Cl_2 \cdot O \cdot CH_3$, (PAL).

2,4-Df: 2,4-dichlorophenole, $C_6H_3Cl_2 \cdot OH$, (obtained through the courtesy of Fil. lic. M. Matell; m.p. $40-41^\circ C$).

All tests were made with young flax seedlings in solution culture (25° C, 6.7 mM phosphate+5 mM Ca-nitrate, pH 5.9) as earlier described (1).

Experimental results

The action curves of the pure substances are shown in figure 1. As is apparent from table 1 the »half repression» or *Hr* values of the substances tested lie within the range found for different auxin antagonists, the inhibiting effects of which are thought to depend on a non-specific toxic action (3, p. 630). The toxic range is not sharply delimited, however, and for Nnm and 2,4-Da at least, the possibility of a very weak auxin effect must be discussed. For 2,4-Df, Ne and NAn the form of the action curves seems to indicate the toxic character, in that the growth rapidly decreases to zero with increasing concentration, while the typical auxin curves usually tend to become almost horizontal at high concentrations. In the highest concentrations of 2,4-Df at least, the roots were apparently dead at the end of the test period. Such phenol effects upon roots have been described and illustrated by Hansen (10). 1-Naphthyl-acetonitrile (NAn) is wholly devoid

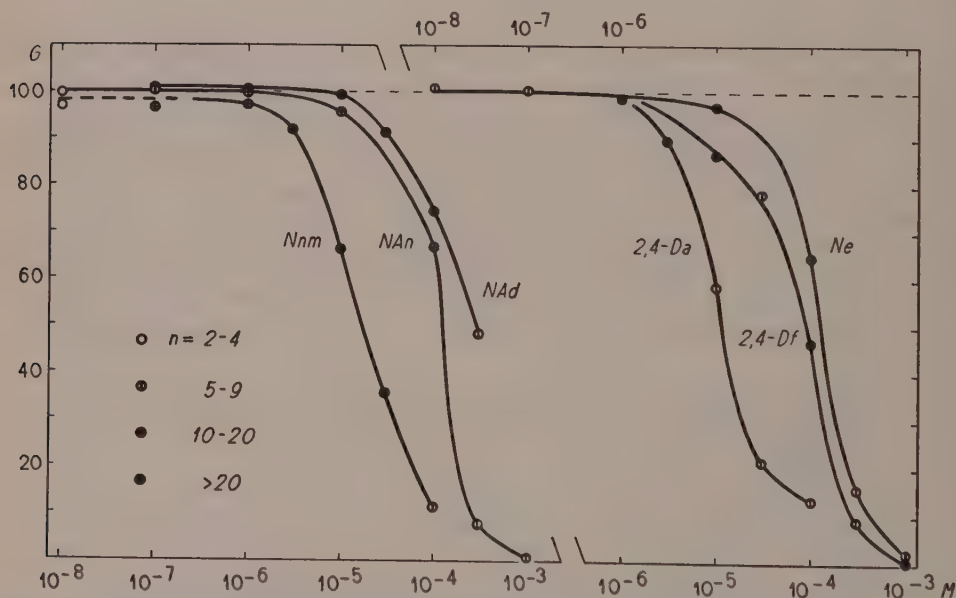


Figure 1. Effects of some non-carboxylic substances on the root growth of flax seedlings. Root growth in per cent of control (*G*) plotted against a logarithmic concentration scale (*M*=mole per litre); *n* is the number of tubes, each with 14 seedlings, represented by the points. *G*-values for low 2,4-Da concentrations: 10⁻⁸ 99.5 (*n*=31), 10⁻⁷ 102.2 (*n*=14), 10⁻⁶ 99.2 (*n*=15). 2,4-Df: 10⁻⁶ 100.8 (*n*=13), 10⁻⁷ 96.6 (*n*=8), 10⁻⁸ 96.9 (*n*=9).

Table 1. *Molar concentrations needed for 50 per cent inhibition of root growth (Hr). The values of the right column have been included for the sake of comparison. Abbreviations: IA=3-indolyl-acetic acid, 2,4-D=2,4-dichlorophenoxy-acetic acid, 1-NA=1-naphthyl-acetic acid, PA=phenyl-acetic acid, 2-NMSeA=2-naphthylmethyl-selenide-acetic acid, 1-NMSA=1-naphthylmethyl-sulfide-acetic acid, (—)-2-NOP=laevorotatory α -(2-naphthoxy)-propionic acid, POiB= α -phenoxy-iso-butyric acid.*

Substance	<i>Hr</i>	Substance		<i>Hr</i>	Ref.
NAd	2.8×10^{-4}	Strong auxins	IA	0.6×10^{-8}	4
Ne	1.3×10^{-4}		2,4-D	3.9×10^{-8}	4
NAn	1.3×10^{-4}		1-NA	6.3×10^{-8}	1
Nf	8.5×10^{-5}	Weak auxin	PA	2.2×10^{-6}	4
Nnm	1.7×10^{-5}	Auxin antagonists	2-NMSeA	2.0×10^{-5}	3
2,4-Df	9.3×10^{-5}		1-NMSA	8.0×10^{-5}	3
2,4-Da	1.1×10^{-5}		(—)-2-NOP	8.0×10^{-5}	2
			POiB	3.2×10^{-4}	4

of activity in the pea test (15), and causes a very slow response in the tomato plant (leaf epinasty), probably after hydrolysis to 1-NA in the living tissue (16).

For *1-naphthylacetamide* (NAd) the situation seems to be much the same as for the acetonitrile. NAd causes responses in many tests which are similar to those caused by 1-NA, but occur more slowly (12). In the *Avena* curvature test it is practically inactive (5, 12). In the pea test it shows some activity (12; 15, p. 145) which is thought by Veldstra (15, p. 143) to be direct and to be mediated by a tautomeric form ($-\text{CH}_2 \cdot \text{C}(\text{NH}) \cdot \text{OH}$). The slow responses in many tests together with the inactivity in the root test makes a conversion hypothesis seem more plausible, however, and different plant parts could certainly have a differing ability to hydrolyse NAd to 1-NA. It should be mentioned that a somewhat lower *Hr*-value (5×10^{-5}) was found in some preliminary tests made in 1950 (the data of fig. 1 are from 1952). It cannot be judged at present, if this is due to a slight variation in the sensitivity of the roots (cf. 2, p. 550), or if perhaps some hydrolysis can occur in the solutions. The present stock solution was made without heating, and no increase in activity could be detected, when it, after being kept in cold storage for a week, was compared with a freshly made solution.

For *1-naphthyl-nitromethane* (Nnm) Veldstra (15, p. 144) has found a slight activity in the pea test (4 per cent of that of IA), and has put this activity in connection with the existence of an aci-form ($-\text{CH} : \text{NO} \cdot \text{OH}$). The activity in the root test adds an argument in favour of this hypothesis, and it was thought worth while to test the ability of an auxin antagonist to counteract the inhibition caused by Nnm (table 2). Though the amount of applied antagonist (1-NMSP) is only one third of that of Nnm, the restora-

Table 2. *The effects of 1-NMSP upon the growth inhibitions caused by Nnm and 2,4-Da. 1-NMSP = α -(1-naphthylmethyl-sulfide)-propionic acid. C = the growth of control roots in mm/17 h. n = number of tubes, each with 14 seedlings, used for each concentration (molarity denoted by an exponential number).*

Exp.	C	n	Root growth, relative values (C=100)				
			3×10^{-5} Nnm + 1-NMSP:		3×10^{-5} 2,4-Da + 1-NMSP:		1-NMSP
			0	10^{-5}	0	10^{-5}	10^{-5}
1	21.2	4	38.5	46.0	23.3	29.0	103.0
2	24.7	4	34.1	42.7	19.4	24.0	106.3

tive effect on the root growth is quite conspicuous, and a further indication of a near relation of the Nnm-activity to the auxin mechanism is thus obtained.

For 2,4-dichloroanisole (2,4-Da) the situation is less clear. It is claimed by Bonner (6) to be an auxin antagonist, though not an ideal one, which inhibits the responses of *Avena* sections to IA. 2,4-Da alone causes in medium concentrations a very slight and perhaps insignificant increase in the growth of the sections. In the root test it behaves very much like Nnm (fig. 1, table 2), and could thus be thought to have a rudimentary auxin activity. As has been shown in a previous communication (4), it is by no means impossible to assume that a weak auxin activity could be combined, in the same molecule, with a weak antagonistic activity in respect to stronger auxins. The higher the concentrations are, however, which are needed to obtain clear-cut effects, the greater caution must be paid to the possible interference of toxic actions of a non-specific type, and such an interference is naturally very troublesome especially in judging antiauxin actions expected to depress growth.

None of the six non-carboxylic substances used in this study gives a significant stimulation of the root growth in pure solution (fig. 1). As the maximum stimulations obtainable even with strong antiauxins are comparatively slight in flax roots (2—4, cf. 8) this is by no means an argument against a weak antiauxin activity. Very slight growth stimulations are difficult to detect with certainty, and could also be masked by the slightest traces of an accessory toxic action. 2,4-D-inhibited roots have turned out to give much more conspicuous results, however, and the substances to be tested were therefore added to 10^{-7} M 2,4-D solutions in amounts up to those just causing a slight inhibition per se. From the results recorded in tables 3—4 it is apparent that the restorative effects on the root growth are very slight, or, in the case of 2,4-Df and Ne, probably absent. The most conspicuous effects were obtained with NAd which could also be applied in the highest amounts, but also for 2,4-Da the effect must be judged significant. The fact

Table 3. *Effects of NAd, NAn, Nnm and Ne upon the growth inhibition caused by 2,4-D. Values presented as in table 2.*

Exp.	C	n	Root growth, relative values (C=100)				
			10 ⁻⁷ 2,4-D + NAd:			NAd:	
			0	10 ⁻⁵	3 × 10 ⁻⁵	10 ⁻⁵	3 × 10 ⁻⁵
3	23.2	4	22.7	25.6	27.1	—	—
4	23.2	4	20.1	21.6	23.1	—	—
5	23.4	4	20.2	23.2	23.4	97.0	89.4
			10 ⁻⁷ 2,4-D + NAn:			NAn:	
			0	10 ⁻⁶	10 ⁻⁵	10 ⁻⁶	10 ⁻⁵
6	24.9	4	24.7	25.1	25.0	99.6	94.8
7	22.7	4	23.4	25.9	26.3	98.0	97.5
			10 ⁻⁷ 2,4-D + Nnm:			Nnm:	
			0	10 ⁻⁶	3 × 10 ⁻⁶	10 ⁻⁶	3 × 10 ⁻⁶
8	26.5	4	23.1	26.0	27.9	104.5	94.4
9	27.7	4	25.3	25.0	26.0	92.4	91.1
			10 ⁻⁷ 2,4-D + Ne:			Ne:	
			0	10 ⁻⁶	10 ⁻⁵	10 ⁻⁶	10 ⁻⁵
10	24.8	4	23.3	25.8	24.8	94.5	88.5
11	23.4	4	25.7	25.4	23.9	98.0	96.1

Table 4. *Effects of 2,4-Da and 2,4-Df upon the growth inhibition caused by 2,4-D. Values presented as in table 2.*

Exp.	C	n	Root growth, relative values (C=100)				
			10 ⁻⁷ 2,4-D + 2,4-Da:			2,4-Da:	
			0	10 ⁻⁶	3 × 10 ⁻⁶	10 ⁻⁶	3 × 10 ⁻⁶
12	21.9	4	14.6	16.4	18.3	96.4	80.6
13	22.9	4	14.4	15.3	15.3	99.6	88.3
14	23.9	4	22.2	21.3	22.2	—	—
15	24.9	4	18.3	18.8	18.2	95.0	91.3
16	23.8	4	18.8	20.2	18.6	98.1	87.9
			5 × 10 ⁻⁸ 2,4-D + 2,4-Da:			2,4-Da:	
			0	10 ⁻⁶	3 × 10 ⁻⁶	10 ⁻⁶	3 × 10 ⁻⁶
17	21.6	4	34.0	38.3	38.2	102.0	88.3
18	22.8	4	32.9	36.0	31.6	99.6	92.8
			10 ⁻⁷ 2,4-D + 2,4-Df:			2,4-Df:	
			0	10 ⁻⁶	10 ⁻⁵	10 ⁻⁶	10 ⁻⁵
19	25.3	4	27.9	27.0	23.2	101.4	82.6
20	25.3	4	25.9	26.9	26.9	101.7	83.9

Table 5. Root growth in 10^{-7} M 2,4-D with additions of various substances. Mean values for the experiments recorded in tables 3—4. Some data from publications 2—4 included for comparison. POA=phenoxy-acetic acid. Data for 1-naphthoxy-acetic acid (1-NOA) from unpublished experiments. For the other abbreviations see tables 1—2.

M	G' = Root growth, rel. values (growth in 10^{-7} 2,4-D = 100)					
	NAd	NAn	Nnm	Ne	2,4-Da	2,4-Df
10^{-6}	—	106	106	105	105	100
3×10^{-6}	—	—	112	—	106	—
10^{-5}	112	107	—	100	—	94
3×10^{-5}	117	—	—	—	—	—
	POA	1-NOA	POiB	(—)-2-NOP	1-NMSP	1-NMSA
10^{-6}	109	172	113	—	140	238
3×10^{-6}	—	—	—	194	210	327
10^{-5}	120	323	159	248	301	411
3×10^{-5}	130	—	319	—	—	—

that it does not increase from 10^{-6} to 3×10^{-6} M 2,4-Da is easily explained by the slight toxic effect, or possibly auxin effect, of the latter concentration.

With the aid of table 5 a comparison of the effects with those of some auxin antagonists of different types, but all containing carboxyl groups, can easily be made. NAd has weaker antiauxin properties even than POA and POiB which are thought to contain »weakly active» ring systems, and in respect to the naphthyl derivatives with free carboxyl groups the difference is yet stronger.

For 1-naphthol (Nf) two combination experiments were reported earlier (1, table 3). 2×10^{-5} M was added to 10^{-7} M 1-NA, and caused an increase in the root growth from 100 to 124. The same amount of 1-NMSP gave an increase to 237, however, and also in this case the carboxy antagonist is thus much more effective. A single experiment with 10^{-5} M NAd added to 10^{-7} M 1-Na (made in 1950) gave no growth restoration (rel. value 96 as compared to 100 for pure 1-NA).

Discussion

In a previous paper (4) the properties of growth-regulating substances have been tentatively described by the use of two different values, the first indicating the affinity of the substance to a protoplasmic bearer (*P*) the other giving an approximate measure of the auxin activity of the molecule when bound to *P*. The substances tested in the present study must be assumed to have a very low affinity which is, in the case of Ne, NAn, NAd, Nf, and 2,4-Df, combined with a total lack of activity ($\text{Aff} < 1$, Act^0). The slow reac-

tions caused by NAn and NAd are thought to depend on their conversion to 1-NA. By analogy, the high activity newly reported (11) for 3-indolyl-acetonitrile (IAN) and 2,4-dichlorophenoxyacetonitrile (2,4-Dn) should perhaps be referred to a rapid conversion of a similar type. As IAN has been reported to be inactive in the pea test (15), it seems necessary to assume a variation in the ability of different plant parts to perform such a conversion.

In the case of Nnm and 2,4-Da some slight activity seems to remain in spite of the absence of a carboxyl group. For Nnm this has been explained by assuming that the slightly acid $=\text{NO} \cdot \text{OH}$ group could fill the functions of the carboxyl group to some extent (15, 14). For 2,4-Da a corresponding explanation is not easily found, and the nature of its inhibiting action upon root growth should be left open till further data on its action are available. We have no precise knowledge as to the function of the acidic group, however, and the possibility thus remains that a rudimentary auxin activity could exist in non-acidic molecules, when the other requirements for activity are well satisfied.

From the present data it seems necessary to assume that the carboxyl group plays an important role in the attachment of the auxin molecules to the active sites, and that it functions in the same way in the auxin antagonists. Otherwise it will be difficult to explain the lack of conspicuous antagonistic activity of the substances now tested. The attachment is thought to depend on the joint function of the ring system and the carboxyl group, and the character of the physiological activity to depend on their steric relation, possibly in combination with other steric factors in the molecule. Such a hypothesis seems to be well compatible with the different types of transformations known to effect a change from strong auxin activity to strong antagonistic activity:

- 1) The insertion of a $-\text{CH}_2\text{-S-}$ or a $-\text{CH}_2\text{-Se-}$ group in the side chain of 1- or 2-naphtyl-acetic acids (1, 3).
- 2) The substitution of both alpha-hydrogen atoms in several phenoxy-acetic acids (7—9).
- 3) The change of the side chain from 2- to 1-position in naphthoxy-acetic acid (cp. table 5; 2-NOA has high auxin activity, $Hr=8.6 \times 10^{-8}$).
- 4) The change of configuration (from dextrorotatory to laevorotatory form) in 2-naphthoxy-propionic acid (2).
- 5) The change from cis- to trans-structure in cinnamic acid (13).

Summary

The effects of some 1-naphthyl and 2,4-dichlorophenoxy derivatives without carboxyl groups on the root growth of young flax seedlings in solution

culture have been studied. No significant growth stimulations could be detected, and the restorative effects on roots inhibited by 10^{-7} M 2,4-D were very slight. It is concluded that the tested substances have a very low affinity for the sites of auxin action, and that the carboxyl group as well as the ring system is active in binding both auxins and auxin antagonists to these sites.

References

1. Åberg, B.: On auxin antagonists and synergists in root growth. — *Physiol. Plant.* 3: 447. 1950.
2. — On the growth substance activity of enantiomorphic α -(naphthoxy)-propionic acids. — *Arkiv f. Kemi* 3 (59): 549. 1951.
3. — The interaction of some auxin antagonists and 2,4-D in root growth. — *Physiol. Plant.* 4: 627. 1951.
4. — On the effects of weak auxins and antiauxins upon root growth. — *Physiol. Plant.* 5: 305. 1952.
5. Avery, G. S., Berger, J. & Shalucha, B.: Comparative activity of synthetic auxins and derivatives. — *Bot. Gaz.* 104: 281. 1942.
6. Bonner, J.: Limiting factors and growth inhibitors in the growth of the *Avena* coleoptile. — *Amer. Jour. Bot.* 36: 323. 1949.
7. Burström, H.: Studies on growth and metabolism of roots. IV. Positive and negative auxin effects on cell elongation. — *Physiol. Plant.* 3: 277. 1950.
8. — Do. VI. The relative growth action of different isobutyric acid derivatives. — *Physiol. Plant.* 4: 470. 1951.
9. — Do. VII. The growth action of α -(phenoxy)propionic acids. — *Physiol. Plant.* 4: 641. 1951.
10. Hansen, Berit: Impurities in technical methoxone and their influence on plants. — *Physiol. Plant.* 4: 667. 1951.
11. Jones, E. R. H., Henbest, H. B., Smith, G. F. & Bentley, J. A.: 3-Indolylacetonitrile: a naturally occurring plant growth hormone. — *Nature* 169 (4299): 485. 1952.
12. Mitchell, J. W. & Stewart, W. S.: Comparison of growth responses induced by naphthalene acetamide and naphthalene acetic acid. — *Bot. Gaz.* 101: 410. 1939.
13. van Overbeek, J., Blondeau, R. & Horne, V.: Trans-cinnamic acid as an anti-auxin. — *Amer. Jour. Bot.* 38: 589. 1951.
14. Thimann, K. V.: Plant growth hormones. — Pincus & Thimann, *The hormones* 1: 5. New York 1948.
15. Veldstra, H.: Researches on plant growth substances, IV—V. Relation between chemical structure and physiological activity, I—II. — *Enzymologia* 11: 97, 137. 1944.
16. Zimmerman, P. W. & Wilcoxon, F.: Several chemical growth substances which cause initiation of roots and other responses in plants. — *Contr. Boyce Thompson Inst.* 7: 209. 1935.

An Action Spectrum for the Light Induced Changes in the Viscosity of Plant Protoplasm

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Introduction

In earlier papers (12, 18) the great response of plant protoplasm to light, as shown by changes of the protoplasmic viscosity, has been demonstrated. A preliminary action spectrum was given (18) showing greatest response of the protoplasm to the blue portion of the visible spectrum. Little or no response could be obtained by using red, green or yellow light.

This paper is a more detailed report of the light response of the protoplasm.

Method

Leaves of *Helodea densa* were used as experimental object. Before the illumination experiments were performed, shoots of this plant were kept in darkness for three days in order to have a constant initial value of the protoplasmic viscosity (18).

The viscosity was determined by the method described earlier (18). Leaves were placed in small tubes filled with culture water and then centrifuged at 3700 r.p.m., corresponding to a centrifugal force of $1225\times g$. The cell contents of leaves thus treated will be suitably stratified after a time of centrifugation of 8—10 minutes.

In order to get light of well-defined wave-lengths and of a high light intensity, interference filters combined with a projection-lamp (Philip 297 G) were used as monochromators. The light intensity of the lamp could be

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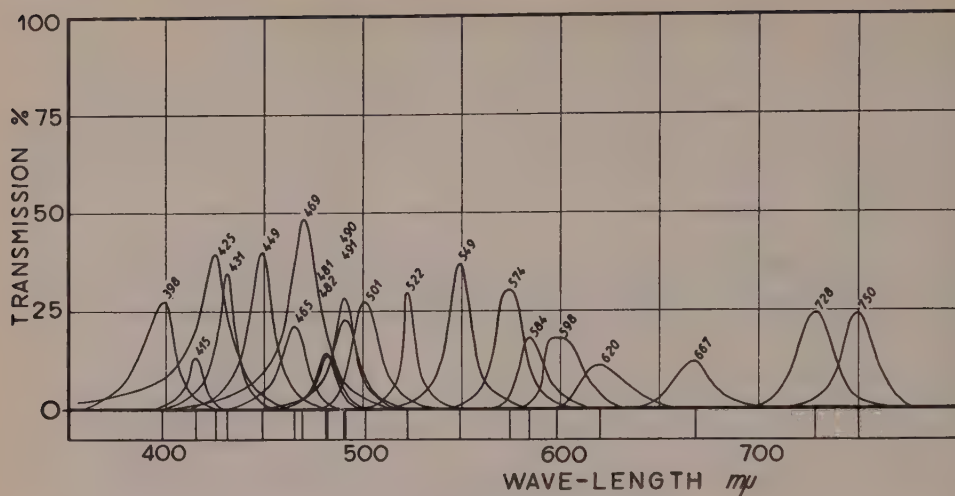


Figure 1. *Spectral transmission of the interference filters.* The curves for the transmission have been obtained by means of a Beckman spectrophotometer. The values for the maximal transmission indicated on the top of the curves, are also indicated by vertical lines along the bottom of the figure.

changed by means of a universal transformer. The transmission curves of the filters used are shown in figure 1. The filters give distinctly isolated spectral areas, with little stray light. Between the lamp and the filters was placed a water cuvette with a continuous change of water plus a heat absorbing glass filter (ON 19, Chance Brothers Ltd., England) according to Virgin (18). By this arrangement transmission of light over about 750 $m\mu$ is eliminated. The interference filters were placed immediately behind a photographic curtain shutter whereby all the light from the lamp passed through the filters. The casing for the lamp and the water filter was quite light proof. The total energy content of the incident light was measured by means of a thermopile according to Moll and Burger (Kipp & Zonen, Delft) combined with an Original Moll Galvanometer in the same manner as described earlier (18). All experiments were performed in a thermostat-regulated dark room at 20° C.

Experimental

The action spectrum of the light-induced viscosity changes of the protoplasm has been determined by means of two different methods.

1. The percentage of affected cells of the whole illuminated area of the leaf was determined after centrifugation. In this case the leaves were

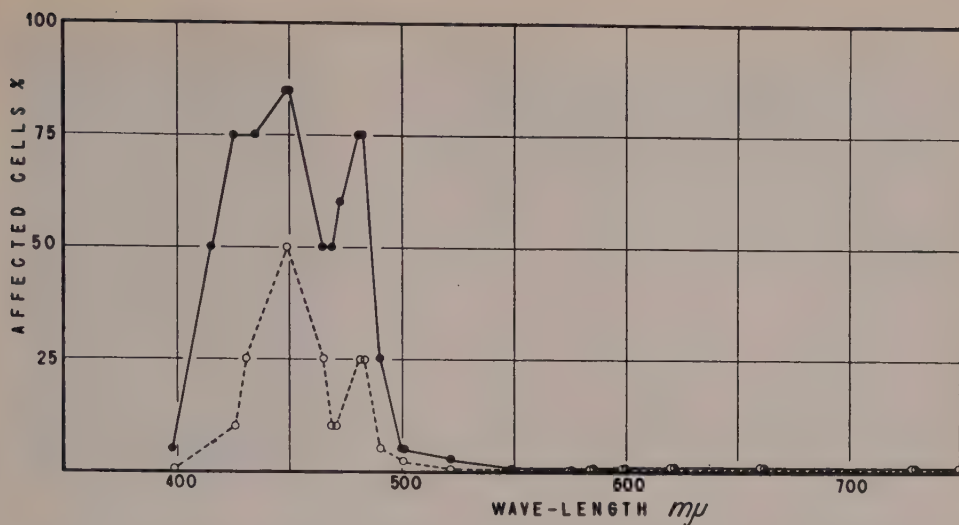


Figure 2. Action spectrum for the light response of protoplasm, determined by using the degree of displacement after centrifugation as an indication of the light-effect. The two curves represent two experiments performed at an interval of three months (June and September, continuous and dotted lines respectively).

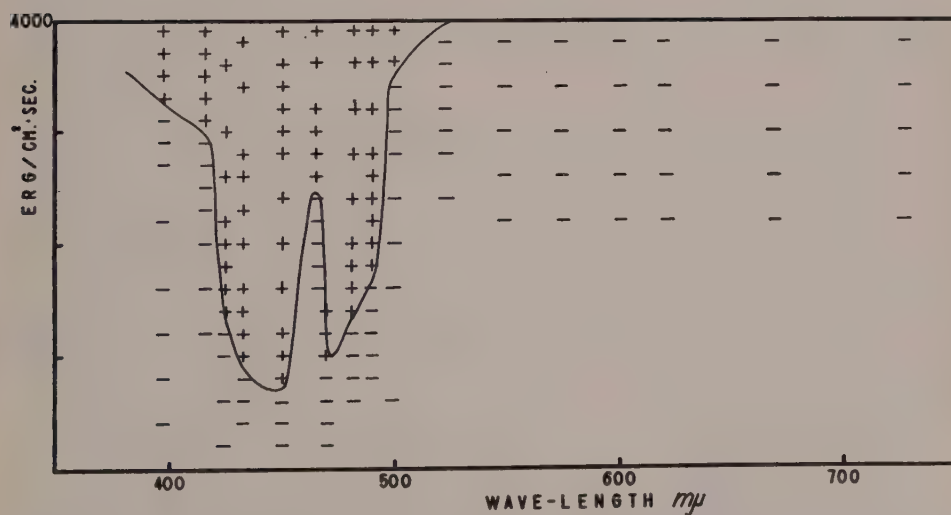


Figure 3. Action spectrum for the light response of protoplasm determined by using the light-energy required to cause a decrease of the protoplasmic viscosity as an indication of the light-effect.

illuminated with the same total energy at the different wave-lengths. The results of these experiments are shown in figure 2.

2. The threshold value necessary for obtaining a decrease in the initial value of the viscosity was determined by varying the light intensity. The results are shown in figure 3.

The experimental procedure for the two methods was as follows: —

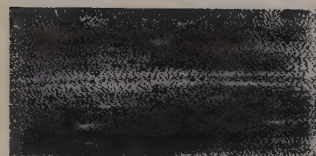
According to the first method the degree to which the cell contents were displaced after centrifugation — the effect of the illumination — is expressed as a percentage of the whole surface of the leaf. It is unavoidable that a certain degree of subjectivity is involved in such a determination since the degree of displacement is estimated by looking upon the centrifuged leaf under a microscope. From figure 4 it is evident, however, that it is possible to observe a gradation in the light effect. In order to be quite sure that variations in the degree of displacement were solely an effect of different responses to illumination and were not due to variations in the initial value of the protoplasmic viscosity, the latter was controlled by repeated measurements of untreated leaves. Earlier (18) it was shown that all the leaves on the same plant, further than 5 cm from the apex had a constant initial value for their protoplasmic viscosity. It is, however, of great importance not to use leaves nearer than 10 cm from the apex because even if they show the same initial values for their protoplasmic viscosity, they can react in different ways to illumination when compared with the leaves lower down. To avoid errors due to changes in the speed of the centrifuge a check consisting of two untreated leaves was always centrifuged together with illuminated leaves. By these arrangements one obtained a constant control of the viscosity state of the experimental material. As a matter of fact no great variations of the experimental material were found.

In the earlier work on this subject it was shown that after short-time illumination with high light intensities giving a decrease of the protoplasmic viscosity as the first light effect, this decrease was maximal three minutes after the beginning of illumination, even if the illumination itself had only lasted for a shorter period of time. In the preliminary work in connection with the planning of the experiments reported in this paper, an illumination for three minutes was shown to give the best results with the light intensities which could be obtained.

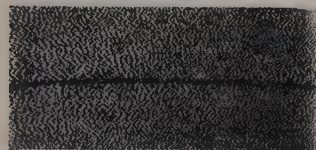
After the illumination, the leaves were immediately centrifuged for two minutes. Many experiments were performed to find the best experimental conditions of observing the effect. If the centrifugal speed is too high or if centrifugation is continued for too long the contents of the illuminated cells will be completely displaced and no differences between differently illuminated cells will be seen. Again, the time of illumination must not exceed a



0



50



100

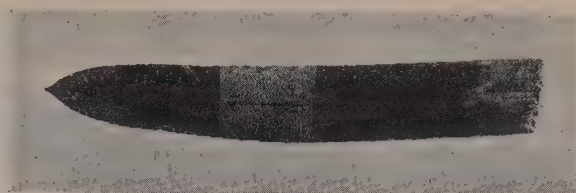


Figure 5. A leaf of *Helodea* which had been illuminated through a small aperture in a piece of tinfoil and then centrifuged.

Figure 4. Different degrees of displacement of the cell contents after centrifugation of a leaf. The three leaves show a percentage of displacement of 0, 50 and 100 respectively. These degrees of displacement have been obtained by centrifuging unilluminated leaves for 2, 6 resp. 9 minutes.

certain value, because it is difficult to get unequivocal results if the cells are illuminated for such a long time that the viscosity begins to show an increase. In experiments where the same light intensities at different wave-lengths are used, this intensity is limited by the filter-glass transmitting the lowest percentage of the incident light. This implies that only small light intensities can be used because some filters for the shorter wave-lengths transmit smaller quantities of energy (cf. figure 1). The light intensities used in these experiments are therefore only slightly higher than those below which the first effect of illumination is an increase of the protoplasmic viscosity according to Virgin (18).

The fact that the light-effect is strictly located to the illuminated cells (17, 18) without any transmission of stimulus to the adjacent areas has been the basic principle for the determination of the action spectrum according to the second method.

When a leaf is illuminated through a small aperture in a piece of tinfoil fitted on a slide and then centrifuged, the localisation of the effect is as shown in figure 5. Owing to the very sharp borderline between the illuminated- and unilluminated areas of the leaf, even small differences in the degree of displacement of the cell contents of the differently illuminated areas can be estimated.

During illumination the slide with the tinfoil was placed vertically and perpendicular to the light beam. It was unnecessary to make any special arrangements to fix the leaf in front of the aperture of the tinfoil, because the wet leaf adheres closely to the slide and is kept in position by the surface tension of the water. After illumination, the leaf was immediately placed in

a glass tube with culture water and centrifuged. In these experiments illumination lasted for 3 minutes and the centrifugation thereafter for 50 seconds. When the leaf had been taken out of the centrifuge it was immediately fixed in a solution of formaldehyde and chromic acid (according to Navashin) in order to prevent subsequent movement of the displaced cell contents. Using this procedure it is possible to obtain clear information as to whether the light in question has had any effect or not. Small variations in the initial protoplasmic viscosity in different leaves are of no consequence by using this method as the control area and the illuminated area are located in the same leaf. By varying the light-intensity at each wave-length, the threshold value required for a response to light was determined over the entire spectrum. The results are shown in figure 3.

It must be emphasized here that the threshold values plotted in this fig. along the ordinate do not represent absolute threshold values for the light response of the protoplasm. They are only the energy values which have to be used in order to have a response under the present conditions (illumination for 3 minutes thereafter centrifugation for 50 seconds). The absolute threshold values are much smaller (18). The main point in this investigation has been to measure the relative values of the response after illumination with different wave-lengths and to obtain a gradation of the effect. The absolute level of the light-energies used is of less importance.

From figures 2 and 3 it is obvious that the maximal response for the light-induced changes in the protoplasmic viscosity is located to the short-wave part of the visible spectrum. In the experiments reported here, no response could be obtained in red light.

The response curves show two maxima, one at 450 $m\mu$ and another between 470 $m\mu$ and 480 $m\mu$. They were well defined in all the repeated experiments performed. The exact position found for the second maximum differed according to the method used. Since the second method described is capable of greater accuracy, the value of 470 $m\mu$ thus obtained is to be preferred.

Discussion

The hitherto known light sensitive reactions of the living plant cell can be classified in three groups, namely reactions related to photosynthesis, to photoperiodism and lastly to phototropism and the light-growth reactions. In addition, there are several unclassifiable responses such as photomorphoses and reactions in cells specialised for certain irritation receptions such as stomatal cells, germinating spores and others. All of these light-sensitive reactions are characterised by a light-absorbing pigment, the absorption curve

of which is more or less responsible for the action spectrum of the reaction in question.

An action spectrum located to the blue part of the visible spectrum is shown by the phototropic reactions (6, 7). Two peaks located to 440 m μ and 475 μ are exhibited. This action spectrum shows a great similarity with that reported in this paper for the light-induced viscosity changes and it therefore seems quite probable that phototropism and these changes of the protoplasmic viscosity are intimately connected with each other, at least as regards the primary light-absorption mechanism.

The pigment active in light-absorption in phototropism has been the subject of conjecture, from considerations of the action spectrum. According to Bünning (2) this pigment may be a compound belonging to the carotene complex, while Galston and his collaborators are of the opinion that it is riboflavin (3, 4, 5). The *Avena coleoptile*, one of the most investigated objects in respect to these reactions contains both of these substances. The asymmetrical distribution of carotene in the coleoptile speaks in favour of riboflavin being the light-absorbing substance, while on the other hand the existence of the two peaks in the action spectrum speaks in favour of carotene. Cf. Reinert (11).

Phototropic phenomena and the light-growth reactions are all connected with changes in the content and distribution of phytohormones. The primary point of action of the light is as yet unknown. There may be either a direct effect on the phytohormones (cf. Galston, 3) or some change in the protoplasm which in its turn affects the phytohormones (Du Buy, 1; van Overbeek, 10).

The above-reported light-induced changes of the protoplasmic viscosity have been established in objects showing no visible phototropic response. Since the action spectra are so similar, it seems possible that the changes in respect to phytohormones in the phototropic phenomena may be preceded by changes of the protoplasm itself in all illuminated cells.

The influence of phytohormones on the protoplasmic viscosity (*e.g.* see Northen, 8 and Stålfelt, 13), as well as on the protoplasmic streaming, 14, 15, 16), shows on the other hand that even the viscosity changes reported in this paper can be a result of a direct light-effect on the active substance brought about through the co-operation of certain sensitizers such as carotene or riboflavin in accordance with Galston (3, 4).

The fact that the content of indoleacetic acid in plants of *Helodea densa* is rather high (9) renders it possible to investigate if the changes in viscosity are in some way connected with changes of the contents of phytohormones.

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Summary

An action spectrum for the light-induced viscosity changes of *Helodea densa* protoplasm is given.

Only the short-wave regions of the visible spectrum between about 390 m μ and 520 m μ have any effect on the protoplasmic viscosity. Within this area two peaks are found, one at 450 m μ , another at about 470 m μ .

A comparison made between this action spectrum and that for the phototropic phenomena argues in favour of a close relationship between the light-induced viscosity changes and the visible phototropic and light-growth phenomena. Probable connections between the two phenomena are discussed.

References

1. du Buy, H. G.: Über Wachstum und Phototropismus von *Avena sativa*. — Rec. trav. bot. néerl. 30: 798. 1933.
2. Bünning, E.: Phototropismus und Carotinoide III. Weitere Untersuchungen an Pilzen und höheren Pflanzen. — Planta 27: 583. 1937.
3. Galston, A. W.: Riboflavin, light and the growth of plants. — Science 111: 619. 1950.
4. — & R. S. Baker: Inactivation of enzymes by visible light in the presence of riboflavin. — Science 109: 485. 1949.
5. — & M. E. Hand: Studies on the physiology of light action. I. Auxin and the light inhibition of growth. — Am. J. Bot. 36: 85. 1949.
6. Haig, C.: The phototropic response of *Avena* in relation to intensity and wave-length. — Biol. Bull. 69: 305. 1935.
7. Johnston, E. S.: Phototropic sensitivity in relation to wave-length. — Smiths. Misc. Coll. 92: 11: 1. 1934.
8. Northen, H. T.: Relationship of dissociation of cellular proteins by auxins to growth. — Bot. Gaz. 103: 668. 1942.
9. van Overbeek, J.: Auxin in marine algae. — Plant Physiol. 15: 291. 1940.
10. — Wuchsstoff, Lichtwachstumsreaktion und Phototropismus bei *Raphanus*. — Rec. trav. bot. néerl. 38: 287. 1941.
11. Reinert, J.: Über die Bedeutung von Carotin und Riboflavin für die Lichtreizaufnahme bei Pflanzen. — Naturwissenschaften 39: 48. 1952.
12. Stålfelt, M. G.: The influence of light upon the viscosity of protoplasm. — Ark. f. Bot. 33 A: 4: 1. 1946.
13. — Effect of heteroauxin and colchicine on protoplasmic viscosity. — Exptl. Cell. Res. Suppl. 1: 63. 1949.
14. Sweeney, B. M.: Conditions affecting the acceleration of protoplasmic streaming by auxin. — Am. J. Bot. 28: 700. 1941.
15. — The effect of auxin on protoplasmic streaming in root hairs of *Avena*. — Am. J. Bot. 31: 78. 1944.
16. — & K. V. Thimann: The effect of auxins on protoplasmic streaming. III. Jour. Gen. Physiol. 25: 841. 1942.
17. Virgin, H. I.: A localized effect of light on the protoplasmic viscosity of plant cells. — Nature 166: 485. 1950.
18. — The effect of light on the protoplasmic viscosity. — Physiol. Plant. 4: 255. 1951.

On the Physiology of *Phycomyces blakesleeanus* Burgeff

I. Mineral Requirements on a Glucose-Asparagine Medium

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The following medium has proved satisfactory for the healthy growth of a great number of fungi:

Glucose	20 g	FeCl ₃ 1 %	0.5 ml
Ammonium tartrate	5 g	ZnSO ₄ 1 %	0.5 ml
KH ₂ PO ₄	1 g	(Thiamine	50 µg — when needed)
MgSO ₄ · 7 H ₂ O	0.5 g	Pyrex-dist. water	1000 ml

(Melin 1946).

Phycomyces blakesleeanus also grows well on this medium. The economic coefficient $\frac{\text{dry weight of mycelium}}{\text{sugar consumed}} \times 100$ was found to be so high that

there seemed to be little evidence for suspecting abnormal metabolic activities. Development was, however, not quite satisfactory; the rather dense mycelial mats that form on the surface of the liquid medium would display a pronounced tendency to curl up at the edges, and the sporangiophores would become more or less bent or curled, very often being sterile and rather short, with tiny drops of liquid at the tips. The generally unhealthy appearance was especially striking when compared to growth on malt agar slants, where sporangiophores would be long and straight, with normal sporangia. These abnormalities would also occur when ammonium tartrate was replaced by other nitrogen sources. Asparagine would give greater yields in dry weight of mycelia (and casein hydrolysate still greater) than ammonium tartrate, but the abnormal morphological characters would persist. The amount of nitrogen given was the same (0.75 g/l) in all cases.

For planned experiments on the metabolism of the organism in question it seemed desirable to have a standard nutrient medium that would secure perfectly healthy and normal growth. The nature of the abnormalities observed, and the fact that they would occur on various nitrogen sources indicated the possibility of insufficient mineral nutrition. The experiments reported in this paper were performed for the purpose of establishing a synthetic medium made up of chemicals of standard C. P. quality that would secure growth without any insufficiency symptoms. No attempt was made to accomplish a critical analysis of requirements for micronutrients under the strictest possible control of ionic composition of the medium; Pyrex glassware was used, not quartz, and the chemicals were not purified. During the latter part of the work the chemicals were tested for purity with dithi-
zone (cf. section on Material and Methods).

Previous investigations

Through the extensive investigations by Schopfer and coworkers (for bibliography cf. Schopfer 1939 and 1943) and Robbins and coworkers (for bibliography cf. Robbins and Kavanagh 1942 and Robbins and Smitt 1945) a great amount of evidence has been gathered on the physiology of *Phycomyces blakesleeana*. However, relatively little attention seems to have been paid by these workers to the mineral requirements of the organism. As a basal culture medium Schopfer generally utilizes a »modified Coon's medium» containing glucose, asparagine, KH_2PO_4 , $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, and thiamine; C. P. chemicals and Jena glassware are used. Schopfer observed no significant difference in yield if glass distilled water, »ordinary» distilled water, or tap water (»eau courante, tres calcaire») was used (Schopfer 1934). The addition of zinc to the medium had no positive effect. In a later paper (Schopfer 1941) a 15 per cent increase in yield was reported when Hoagland's »A-Z mixture» of trace elements was added to the standard medium. The effect of addition of sea water has also been investigated (Schopfer and Utiger 1941). A considerable increase in growth was found to be the result, both at suboptimal and optimal vitamin B_1 levels (pyrimidine+thiaxole):

Standard medium (optimal B_1)	47 mg
0.1 ml sea water added per flask	85 mg
0.5 ml » » » » »	101 mg
1.0 ml » » » » »	121 mg
(20 ml nutrient solution per flask, 11 days culture)	

The influence of a mixture of ions — Fe, Zn, Cu, Mn, Mo, B, and Ga — upon biotin production is described in a later paper (Schopfer 1943). Biotin production is stimulated; the figures given for »poids thalle» also indicate that there is a consistent though small positive effect on mycelial growth:

Control cultures (excess B_1)	67 —70.5 mg
trace elements added	72 —76.5 mg
Control cultures (slightly insufficient B_1)	57.5—66 mg
trace elements added	66 —77 mg

The very pronounced positive effect of sea water on growth observed in the investigation cited above is ascribed by the authors to the mineral content — »catalyseurs minéraux» — and the view is expressed that the trace elements influence the utilization of organic nutrients and the vitamin. »Nous constatons que les cultures témoins, sans eau de mer, même avec la dose optimale de facteur de croissance, donnent des récoltes faibles. . . on peut conclure que la solution minérale constituée par l'eau de mer exerce une action très favorable, à la condition que la quantité élevée de NaCl soit tolérée par l'organisme. . . Les autres éléments se trouvent à des doses telles que seule une action catalytique peut leur être attribuée. Leur action se manifeste par une meilleure utilisation des constituants organiques du milieu, ainsi que de la vitamine.»¹ In spite of these observations, in his later works — the excellent monograph on »Plants and Vitamins» (Schopfer 1943 and 1949), and a survey article (Schopfer 1945) — Schopfer does not include any trace elements in the nutrient solution recommended for assays on vitamin B₁ with *Phycomyces* as test organism.

Robbins and Kavanagh (1938) include »a modification of Hoagland's A-Z mixture» (Fe, Cu, Mn, B, Li, Al, Sn, Ni, Co, Ti, I, Br) in their nutrient solution for *Phycomyces*. (In earlier investigations by Robbins no trace elements were added.) In later works, Robbins always adds the following »supplementary elements»: Fe, Zn, Cu, Mn, Mo, and B. Ga is included in the list from 1940 on; Steinberg has demonstrated the effect of this element on growth of *Aspergillus niger* (Steinberg 1938). Robbins and his coworkers have, however, not gone very far into the problem of the essentialness of the trace elements added. Robbins and Kavanagh (1941) state that »Although some differences in growth were noted in the various solutions, the addition of trace elements to the nutrient solutions used did not appear to be a factor in determining the thiazole effect» (i.e. the positive effect of addition of excess thiazole). The only report by Robbins and coworkers on quantitative experiments on the influence of trace elements on growth of *Phycomyces* familiar to the author is a paper (Robbins and Hervey 1944) concerning the relative effect of the elements listed above, and of manganese alone, on growth of *Phycomyces blakesleeanus*, *Aspergillus niger* and *Pythiomorpha gonapodyides*. Here the following figures are given for dry weights of *Phycomyces* in the third and fourth successive passages in a basal medium, and in the same medium plus manganese or a mixture of mineral supplements: (Fe, Zn, Cu, Mn, Mo, B, Ga).

Passage 3: Basal medium alone	104.9
0.002 p.p.m. Mn added	123.1
All minerals added	108.1
Passage 4: Basal medium alone	117.5
Mn added	122.8
All minerals added	147.1

The authors conclude that the addition of the mixture of mineral supplements, or of manganese alone, has relatively little effect on *Phycomyces* (while the other two fungi tested display a pronounced positive effect; *Pythiomorpha* will not grow at all without manganese).

Burgeff (1934) criticizes the substrate employed by Schopfer (»— ein sehr unvollständiges kümmerliches Substrat»), and recommends the addition of iron and cal-

¹ Italicized by me. K. Ø.

cium; he does not carry out any experimental comparison, however, between Schopfer's substrate and his own.

In fact, the method and the substrate recommended by Schopfer for the Phycomyces assay on vitamin B₁ has been employed successfully by a number of authors for a wide variety of natural substances (for literature references see Bang 1944 and Schopfer 1945). Meiklejohn (1937) found that it gave good results for blood, and claimed to have proved that substances present in blood other than vitamin B₁ do not interfere. This was refuted by Sinclair (1938 and 1939), who found that an »adjuvant factor» in blood would interfere, most often giving too high values for vitamin B₁. He found that any of the ions Ca, Fe, Cu, and Mn would bring about an increase in yield; sodium pyrophosphate also augmented growth. However, factors in the »adjuvant factor» complex other than the metal ions are considered to be of greater influence.

Leonian and Lilly (1940 a) added the »A-Z modification» employed by Robbins and Kavanagh to their culture solution. This mixture contains no Zn, Mo, or Ga. In a later work (1940 b) they investigate the effect of addition of Fe, Zn, Cu, Mn, Mo, and Ga, singly or in combination, to a medium already containing the »A-Z mixture». Of the ions tested, Zn alone had a positive effect under these conditions. When the nutrient solution was treated with calcium carbonate to remove traces of heavy metals, Fe and Zn proved essential for growth. Neither of them would induce increased growth when added alone, but in combination they effected optimum growth. For zygospor formation the ions Fe, Zn, Mn, Mo, and Ga had no specific effect (Leonian and Lilly 1940 c).

Thren (1941) found no increase in dry weight yield of mycelia after addition of Hoagland's original »A-Z» (which contains Zn, but no Fe, in contrast to Robbins' and Kavanagh's modification). It has been mentioned before that Schopfer, in a contemporary investigation (1941), got a 15 per cent increase in yield with »A-Z».

Lehmann and Nielsen (1941) found that growth of Phycomyces was »wesentlich stimuliert» through addition of Ca and Fe in combination. They give no figures, but the diagram indicates an increase of about 15 per cent.

Lohmann and Cheng (1940) observed that Mg, which is essential for Phycomyces, can be partly replaced by Mn.

Several authors have given evidence for the fact that maximum growth of Phycomyces in the presence of optimum amounts of vitamin B₁ may be considerably greater on natural substrates than on the synthetic medium usually employed (cfr. the »adjuvant factor» of Sinclair). This is often thought to be due mainly to the nitrogen content of the natural substrate, but trace elements may also play a role. Haag and Dalphin (1944) assert that the Phycomyces assay meets with serious difficulties when applied to natural materials (»milieux biologiques»): »Le poids sec du champignon obtenu sur le milieu de base auquel on a incorporé le milieu biologique peut, en effet, être considérablement supérieur à celui contenant seulement un excès de vitamine B₁ pure. Ainsi avec le milieu de base enrichi de l'extraits de levure, nous avons obtenu des poids sec dix fois plus grands qu'avec le milieu de base à excès d'aneurine.»

The »compensation method» of Kocher (1944) is an attempt to overcome this obstacle: The vitamin present in the substance to be tested is removed by adsorption by active carbon, and a comparison is made between growth on a medium containing this carbon-treated substance+known amounts of thiamine and that on a medium containing the untreated substance. Kocher found that this method would give more

reproducible results and generally somewhat lower vitamin B₁ values than the direct method of Schopfer. — The compensation method can not be applied to all media, however, — other substances than the vitamin may be removed by the active carbon treatment, thus making the composition of the media for the test and proof series different so that the intention of the compensation method may not be fulfilled.

Bang (1944) in his important investigation on vitamin B₁ content of blood employs the method and the substrate of Schopfer (Meiklejohn's modification), but stresses that »the values thus found must not be considered as absolutely representative of the vitamin B₁ content, but only as a sort of vitamin B₁-'equivalents' or 'indicators'».

Hurni (1945) investigated the effect of the following mixture of ions: Fe, Zn, Cu, Mn, B, Ni, Co, Ti, and I. With a glucose-asparagine medium the positive effect is only about 10 per cent; with ammonium citrate as a nitrogen source however, the augmentative effect may be as high as 150—200 per cent. When the distilled water for the nutrient solution is pretreated with active carbon, trace elements are given off to the water, causing a considerable increase in yield on an ammonium citrate medium.

1. Basal medium	46.6 mg
2. Trace elements added	64.4 mg
3. As 1, dist. water treated with 15 % carbon	62.5 mg
4. As 1, dist. water treated with 2 % carbon	61.4 mg

Hurni considers the difference between asparagine and ammonium citrate media in response to addition of trace elements to have a direct bearing on the difference in type of nitrogen compound given. He does not take into consideration the possibility that the asparagine might be more contaminated with traces of metals than the ammonium citrate.

Utiger (1947) claims to have established grave discrepancies between estimated values of vitamin B₁ obtained by two different methods, viz. the purely chemical thiocrome method and the Phycomyces method, the latter usually giving much higher values. He claims that the growth is so greatly influenced by interfering factors (»Täuschungsfaktoren») that the method is useless for many natural products. Trace elements play an important role in this factor complex; ash of onions was proved to augment growth.

From the works cited above it will be seen that quite a number of observations has been made on the effect of various trace elements, singly or in combination, on growth of Phycomyces. The results are, however, sometimes contradictory. *There hardly seems to be sufficient evidence for the establishment of a synthetic medium of known ionic composition, suited for vitamin assay or physiological experiments, that will secure growth and development equivalent to that on a natural substrate with an unknown content of ions.*

The augmentative effect of *calcium*, reported by several authors, seems to be of special interest. This element has received relatively little attention in the study of nutritional requirements of fungi. Stiles (1946) states that »the general opinion at present appears to be that this element is necessary for growth of some species but not of others.» The necessity of further experimental work is stressed. Recent review articles (Perlman 1949, Steinberg 1950) and monographs (Foster 1949, Lilly and Barnett 1951) indicate that the amount of evidence brought forth during the last few years is limited; no further experiments with Phycomyces are reported. The most important contribution to the knowledge of the effect of calcium is that of

Steinberg (1948, 1950) which confirms that some fungi require Ca while others do not. Older observations (Davis, Marloth, and Bishop 1928) reporting a positive effect of Ca upon growth of *Aspergillus niger* are definitely refuted by later work (Mann 1932, Rippel and Stoess 1932, and Steinberg l.c.). Steinberg has observed (1946) that Ca will not augment growth of *Aspergillus niger* on Mg-deficient (or better, Mg-insufficient) media when other nutrients are given in optimum amounts; in the presence of *excess* of macronutrients, however, addition of Ca will give increased growth on Mg-insufficient media. This effect, called »pseudosubstitution», is attributed to »amelioration of inhibitions (ion antagonism)». The positive effect of Ca on various fungi observed by Rippel and Stoess (l.c.) was also considered by the authors to be the result of an antagonistic effect of Ca on excess Mg. *Aspergillus niger* did not respond to Ca even in the presence of great excess of Mg. The non-essentiality of Ca to this organism must be considered as an established fact, even if some authors working with *A. niger* still add Ca to the nutrient solution (Mulder 1948).

In addition to the species investigated by Steinberg (1948, 1950), viz. *A. niger*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *Cercospora nicotianae*, *Fusarium oxysporum*, *Pythium irregulare*, and *Thielaviopsis basicola*, a few other fungi have been reported to display a positive response to Ca: *Trichophyton interdigitale* (Mosher, Saunders, Kingery, and Williams 1936), *Marasmius* spp. (Lindeberg 1944), *Psalliota bispora* (Treschow 1944), two *Chaetomium* spp., two *Humicola* spp. and *Trichoderma viride* (Omvik 1951), and several *Tricholoma* spp. (Norkrans 1950). *Chaetomium* spp., particularly *Ch. globosum*, requires Ca for normal fruiting (Basu 1951). Increased penicillin production after addition of Ca has been observed in a strain of *P. chrysogenum* (Jarvis and Johnson 1947).

Evidently calcium deserves more attention as a nutrient for fungi than is generally paid to it.

Material and Methods

For the first six experimental series a +strain of *Phycomyces blakesleeana* kept in stock culture at the Institute of Physiological Botany in Uppsala was utilized, for the latter five series a new strain was obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland. Stock cultures were kept on malt agar slants. When an augmentative effect of calcium upon growth and sporangia formation had been observed, CaCl_2 (0.2 g/l) was added to the malt agar. It was found that this seemed to have a slight stimulative effect; however, formation of sporangia was abundant even without this addition.

For the nutrition experiments the following technique of inoculation was developed. From a stock culture, not less than three weeks and not more than three months old, the sporangia are pulled off with a platinum needle and transferred to sterile distilled water (ca. 50 ml) in an Erlenmeyer flask. After having been shaken the spore suspension is counted roughly in a haemocytometer. The spore suspension is used for inoculation of samples of ca. 150 ml (in 300 ml Erlenmeyer flasks) of the following nutrient solution:

Glucose	20 g	FeCl_3	8.14 mg
Asparagine	3.5 g	ZnSO_4	8.90 mg
KH_2PO_4	1 g	Thiamine	50 μg
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5 g	Dist. water	1 liter

The amount of spore suspension used is varied so as to give inoculation densities from ca. 50,000 to ca. 1.5 million spores per flask, 8 to 10 flasks being inoculated simultaneously. After 2 days at 25° C the spores (or a small percent of them) will have germinated and grown into small submerged mycelia. Shaking of the flask will distribute the single-spore mycelia evenly throughout the liquid; they do not tend to stick together. These mycelia are used for inoculation in the following way: 8 cm watch glasses, thoroughly washed, are placed in 10 cm Petri dishes; 8 to 10 dishes are wrapped in paper and dry-sterilized. From a suitably dense culture a few ml of the liquid with some suspended mycelia is poured into the watch glass in a Petri dish, and the cover is replaced. The dish is placed on a dark background and illuminated sideways by means of a microscope lamp; the mycelia will then be clearly visible. If there is a suitable number of mycelia in the watch glass, they can easily be examined with a low-power lens (Zeiss Kopflupe) and picked up singly with a glass needle. (A convenient needle is easily drawn in the flame of a Bunsen burner from a 2 or 3 mm glass rod.) The single-spore mycelia are transferred to culture flasks — each flask is thus inoculated with one mycelium, mycelia of the same size (approximately) being chosen. Most often 4 to 10 mycelia can be taken from the same watch glass. When no more mycelia of correct size can be found in the batch, the watch glass is discarded and a new sample of the culture is poured into a fresh watch glass.

The nutrient solution used for the experiments had the following composition (save trace elements):

Glucose	30	g
Asparagine	3.5	g
KH ₂ PO ₄	1.0	g
MgSO ₄ · 7 H ₂ O	0.5	g
Thiamine	50	µg
Dist. water	1	liter

This solution will be called »A 30». For experiments nos. 1—3 the amount of glucose used was 20 g (»A 20»). For experiment no. 1 ammonium tartrate — 5 g — was used instead of asparagine (»B 20»); this compound was also used in some series in later experiments, eventually with 30 g glucose (»B 30»).

The trace elements whose effects were investigated were the following:

Ca	—	given as	CaCl ₂
Fe	»	»	Fe ₂ Cl ₆ · 6 H ₂ O
Zn	»	»	ZnSO ₄ · 7 H ₂ O
Cu	»	»	CuSO ₄ · 5 H ₂ O
Mn	»	»	MnSO ₄ · 4 H ₂ O
Mo	»	»	(NH ₄) ₂ MoO ₄ · H ₂ O or Na ₂ MoO ₄ · 2 H ₂ O
B	»	»	Na ₂ B ₄ O ₇ · 10 H ₂ O
Ga	»	»	Ga ₂ (SO ₄) ₃ · 18 H ₂ O

For convenience even Ca will be called a trace element, although it is given in comparatively large amounts. In the first four experiments the trace elements were given in varying amounts, until the proper concentrations were found; therefore the concentrations used will be given for each experiment separately.

Redistilled water, the second time distilled in a Pyrex still, was used throughout. The glassware was Pyrex; it was rinsed with bichromate-sulphuric acid, washed thoroughly with hot water, distilled water (three times), and glass-distilled water.

For experiments 7 to 11 the macronutrients (i.e., the constituents of the basal medium) and the distilled water were tested with dithizone (Fischer 1934, 1937, Snell and Snell 1936, Stout and Arnon 1939, Sandell 1944, Feigl 1947). The test was carried out in the following way: 4 mg dithizone (diphenylthiocarbazone) were dissolved in 100 ml chloroform, and 0.2 ml of this solution were shaken vigorously with 2 ml of the solution to be tested in a small test tube (100 mm×9 mm, cubic capacity ca. 4 ml). The tube must always be tested for purity with dithizone+glass-distilled water immediately prior to use. At the pH of the nutrient solution, ca. 5, this test is extremely sensitive to several heavy metals, including Cu and Zn. The dithizone test is discussed further in some detail on p. 605.

The culture media were autoclaved at 120° C for 20 minutes. pH was measured electrometrically with a glass electrode (instrument from Radiometer, Copenhagen). There were either 10 ml of liquid in a 125 ml Erlenmeyer flask or 15 ml in a 300 ml Erlenmeyer; both gave a liquid depth of 2—3 mm. It was found that a small depth (and correspondingly, a large surface area) was favourable for rapid growth; also the agreement between parallels was best under these conditions. The cultures were kept at 25° C; they were usually harvested after 2, 3, and 4 days. In some experiments, the harvest was taken after 3 and 4 days, in a few only after 4 days. The number of parallels will be given for each experiment separately. Mycelia were dried at 110° C for 8—12 hours before weighing. The sugar content of the culture liquid after harvest was determined according to Bertrand (in experiments 8—11).

When comparing the mycelial weights obtained in this investigation with those of other investigations on the same organism, one should bear in mind that the mycelia were pre-grown for two days before they were planted. Also the small depth and large surface area speed up growth, probably for two reasons: gas exchange between liquid and air is facilitated, and the small depth causes the submerged mycelia to come into early contact with the surface so that aerial hyphae can be formed. In orientation experiments with different depths it was found that growth rate increased greatly as soon as this contact was achieved.

Culture experiments

Experiment 1. For this experiment solution B 20 (ammonium tartrate as N-source, 20 g glucose — cf. p. 589) was used as basis. Trace elements were given in the following amounts: Ca, 20 p.p.m., Fe, 1.6 p.p.m.; Zn, 2.2 p.p.m.; Cu, 0.125 p.p.m.; Mn, 0.5 p.p.m.; Mo, 0.5 p.p.m.; B, 0.1 p.p.m. Fe and Zn were always given in combination — called »FeZn» — although from separate stock solutions. Cu, Mn, Mo, and B were given in combination — »CuMnMoB» — from a mixed stock solution containing all four elements. The following combinations of ions were employed:

No. 1: B 20+FeZn

» 2: » +FeZn+Ca

» 3: » +FeZn+CuMnMoB

» 4: » +FeZn+CuMnMoB+Ca (all)

Table 1. Experiment 1. *Growth of Phycomyces blakesleeanus on ammonium tartrate media of varying ionic composition. For further explanation see text.*

No.	pH start	Dry weight of mycelia					pH 4 days
		2 days	3 days		4 days		
		mg	mg	0/0	mg	0/0	
1	5.3	3.8	22.25	59.6	39.65	63.9	3.8
2	5.3	7.7	31.6	84.0	56.3	90.6	3.5
3	5.4	4.2	25.4	67.6	50.2	80.8	3.6
4	5.4	9.5	37.6	100	62.1	100	3.4
5	6.6	9.7	40.3	107.2	66.2	106.6	3.4
6	5.4	6.1	29.1	77.4	55.2	88.9	3.4

No. 5: same as 1, but tap water instead of distilled water.

- » 6: B 20 + FeZn + soil ash: a sample of soil was ashed, the ash dissolved in hydrochloric acid, (without being weighed beforehand), neutralized with potassium hydroxide, and 2 ml added to 250 ml nutrient solution.

The series were made up in quadruplicate; 125 ml flasks with 10 ml medium were used. Mycelia were harvested after 2, 3, and 4 days. The effect of addition of Ca (2) to a medium already containing Fe and Zn (1) is striking. Addition of the combination CuMnMoB also increased growth considerably, especially when Ca was absent (3) but also in the presence of Ca (4) there is pronounced augmentation. Tap water, with added Fe and Zn (5), is superior to all artificial ion combinations tested. Soil ash (6) in addition to Fe and Zn also has a clear augmentative effect. The relative yields are calculated in percent of the yield on the best artificial medium.

In addition to the difference in yields there were also pronounced morphological differences between the series. The mycelia that had received Ca showed a much less pronounced tendency to curl up at the edges, and sporangiophores were long and straight with well developed young sporangia, while the Ca-deficient mycelia all displayed the symptoms described in the introduction. The cultures on the tap water medium were also very well developed, without any deficiency symptoms. The soil ash cultures were intermediate in appearance. The tap water (from the municipal waterworks of Uppsala) is very rich in Ca: ca. 80 mg Ca^{2+} per liter (personal communication from Dr. G. Lohammar, Uppsala). It seems very likely that the augmentative effect of tap water should for a great part be ascribed to its content of Ca. The ion combination CuMnMoB alone had no effect on development of sporangiophores and sporangia.

Experiments 2 and 3. In these two orientation experiments an investigation was made on the effect of addition of different combinations of ions

to basic media with asparagine as the nitrogen source (A 20, cf. p. 589), made up either on distilled water or on tap water (called t.w.). For comparison one series was included where the amount of glucose was increased to 30 g (A 30) and two series with ammonium tartrate as the nitrogen source (B 20). The ion combinations employed are given in table 2. In experiment 3 three different brands of glucose were compared, viz. a purissimum quality from the Swedish firm Kebo (in the table called Ke), a C.P. quality from Rahway (Rah), and a C.P. quality from Coleman and Baker (C.B.). In experiment 2 the Kebo glucose was used throughout. Trace elements were given in the same amounts as in experiment 1, with an exception for series 10, 11, 12, 19 and 20 where the amounts were one tenth of those in experiment 1 for all trace elements except Ca — of this element 20 ppm. were always given. In series 12 a little extra Cu was added, making the total concentration 0.05 ppm., as against 0.0125 ppm. in series 10, 11, 19, and 20, and 0.125 in the other series.

Ten ml nutrient solution in 125 ml flasks were used in all series. pH was adjusted to ca. 5. The series of experiment 2 were made up in duplicate, and the cultures were harvested after 2, 2 $\frac{1}{2}$, 3, 3 $\frac{1}{2}$, and 4 days. In experiment 3 triplicate series and harvest after 2, 3 and 4 days were employed.

The main results are compiled in table 2. No absolute values are given, but relative yields in percent of those on the »complete» media A 20 + CaFeZnCuMnMoB (nr. 8 and 9). Unfortunately no direct comparison can be made between the series of expt. 2 and those of expt. 3, as Kebo glucose was used in the reference (complete) medium in the former experiment (series 8), while Rahway glucose was used in the corresponding medium in the latter experiment (series 9). The only medium which is common to both experiments is nr 2 (A 20 + Ca, Ke), but this medium is so incomplete in ionic composition that it is not suited as a reference medium. Therefore the relative yields are computed for each experiment separately, in percentages of series 8 for expt. 2, and of series 9 for expt. 3. Only the values for 3, 3 $\frac{1}{2}$ and 4 days have been taken into consideration, the values for 2 and 2 $\frac{1}{2}$ days being less reliable, although they fall in well with those of the later harvests. For experiment 2 the percentage values for 3 and 3 $\frac{1}{2}$ days were computed separately, but in the table their arithmetic middles are given. It will appear from the table that there is generally good agreement between the relative yields of the 3-day harvest and those of the 4-day harvest, except for the complete media 8—12 and 16—20. In these series the differences observed in the 3-day harvest seem to disappear in the 4-day harvest. The reason for this is, however, that the amount of glucose was insufficient, causing growth to stop at 80—85 mg dry weight; the medium will then be almost entirely depleted of sugar. With 200 mg sugar given (and consumed)

Table 2. Experiments 2 and 3. *Relative growth* (in per cent) *on asparagine media with three varying brands of glucose* (Ke=Kebo, Rah=Rahway, C.B.=Coleman and Baker), *made up either on distilled water or on tap water (t.w.), and with varying additions of microelements*. Basic medium A 20. For further explanation see text.

No	Medium; type of glucose, if tap water, ions added	Mean of 3 a. 3 1/2 d.	3 days		4 days	
		Expt. 2	Expt. 3	Expt. 2	Expt. 3	
1	Ke, basic medium	54.8			56.0	
2	Ke, +Ca	76.3		88.0	67.0	84.3
3	Rah, +Ca			73.6		71.3
4	C.B., +Ca			81.1		78.4
5	Ke, +FeZn	56.8			60.0	
6	Rah, +CaFeZn			103.3		98.2
7	Ke, +FeZnCuMnMoB	52.4			60.0	
8	Ke, +CaFeZnCuMnMoB	100			100	
9	Rah, +CaFeZnCuMnMoB			100		100
10	Ke, +Ca + 1/10 FeZnCuMnMoB	111.4			103.0	
11	Rah, +Ca + 1/10 FeZnCuMnMoB			105.9		101.1
12	as 10, but extra Cu	112.1			102.0	
13	Ke, t.w. — basic medium			70.2		67.1
14	Rah, do.			63.8		55.3
15	C.B., do.			70.4		62.7
16	Ke, t.w., +FeZn	121.3			102.5	
17	Rah, t.w., +FeZn			110.7		98.4
18	Rah, t.w., +FeZnCuMnMoB			123.9		97.1
19	Rah, t.w., + 1/10 FeZnCuMnMoB			130.3		95.2
20	as 19, but added Ca			137.1		97.5
21	Basic med. less Mg, Ke, t.w.	48.8			20.9	
22	30 g glucose Ke, t.w. + FeZn	126.0			119.0	

per flask (10 ml solution) and a yield of 80—85 mg the economic coefficient will be over 40 per cent. When the amount of glucose was increased to 300 mg per flask, dry weight increased to 96.8 mg (series 22). Evidently at least 300 mg is necessary to secure maximum growth; with only 200 mg no differences can be observed between media of optimum and nearly optimum mineral composition. For these media comparison must therefore be based on the 3-day harvest only.

In addition to the *quantitative* effects of the different ions that appear from table 2, the following observations were made on the *qualitative* effects on growth and morphological development:

On the »complete» synthetic media (series 8—12) growth and development was normal and vigorous; mycelial mats were only slightly arched, and sporangiophores were numerous, long and straight and almost 100 per cent fertile; no deficiency symptoms whatever could be observed. Omission of Ca called forth the same deficiency symptoms as those observed on corresponding ammonium tartrate media; sporangiophores were for a great part bent or curled, many of them being sterile, and the plechtenchymatic surface mats

had a strong tendency to curl up, even more so than on ammonium tartrate. On tap water media, with a natural content of ca. 80 ppm. Ca, development was normal without any further addition of Ca. (The slight positive effect on yield of addition of Ca — compare 19 and 20 — seems highly questionable). — Omission of *FeZn* had a very pronounced effect on morphological development, in tap water media as well as in distilled water. All series where *FeZn* was left out — series 1, 2, 3, 4, 13, 15 — displayed the same deficiency symptoms; formation of sporangiophores and sporangia was entirely inhibited; a cadmium yellow, somewhat brittle, and rather thin surface mycelium formed that was almost bare, covered only by a very short, velvety, whitish fur (less than one mm), with single coarse, black, hairlike, sterile aerial hypha. — The ion combination *CuMnMoB* had no effect on morphological development. — When *Mg* was omitted from a tap water medium (series 21) growth stopped after $2\frac{1}{2}$ —3 days, and development was very poor; evidently the tap water does not contain sufficient *Mg* to secure normal growth. In preliminary experiments (not reported here) it had been observed that no growth whatever would take place when *Mg* was omitted from a distilled water medium. — The *tap water* had no effect on morphological development, except for that which must be ascribed to its Ca content.

The following preliminary conclusions can be drawn from experiments 2 and 3:

1: Either Fe or Zn, or both of them, are necessary for normal development, and have a strong augmentative effect on dry weight yield.

2: Ca has a positive effect on morphological development, especially on sporangiophore formation. It also increases yield considerably.

3: On synthetic media (distilled water) no effect could be observed upon growth or morphological development of the ion combination *CuMnMoB*. On tap water media yield was somewhat increased when these ions were added to a medium containing Fe and Zn (compare 17 and 18).

4: A decrease in concentration of trace elements (save Ca) seemed to be slightly favourable (series 10, 11, 12, 19, 20).

5: Tap water seemed to have an augmentative effect on dry weight yield in addition to that which must be ascribed to its Ca content.

Experiment 4. The purpose of this experiment was a comparison between distilled water media with varying amounts of Ca, and tap water media with and without extra Ca. 30 g glucose per liter were given (A 30), and a complete mixture of trace elements proper in the following amounts: Fe, 0.32 p.p.m.; Zn, 0.44 p.p.m.; Cu, 0.05 p.p.m.; Mn, 0.1 p.p.m.; Mo, 0.1 p.p.m.; B, 0.02 p.p.m. These concentrations are one-fifth of those employed in experi-

Table 3. Experiment 4. *Growth on distilled water media — d.w. — with varying additions of Ca, compared with tap water media — t.w. — (natural content of Ca 80 p.p.m.), with or without added Ca. Basic medium A 30+FeZnCuMnMoB, see text.*

No.	Medium; composition	Dry weight of mycelia					pH after 4 days
		2 days mg	3 days		4 days		
			mg	0/o	mg	0/o	
1	D.w.+Ca, 4 p.p.m.	19.6	68.1	100.2	90.8	96.6	3.2
2	» » 20 p.p.m.	15.0	68.0	100	93.7	100	3.0
3	» » 100 p.p.m.	17.1	70.4	103.5	99.4	106.1	2.7
4	T.w., Ca cont. 80 p.p.m.	—	72.8	107.1	97.6	104.2	2.9
5	As 4, but added further Ca, 60 p.p.m. (total 140)	16.3	73.0	107.3	100.8	107.6	2.7

ments 1—3, except for Cu, where two-fifths were employed; for convenience these amounts will be designated as »Fe», etc., not as » $\frac{1}{5}$ Fe», etc. The varying amounts of Ca given will appear from table 3. The pH was adjusted to ca. 5. Series were made up in triplicate, 10 ml in 125 ml flasks. Cultures were harvested after 2, 3, and 4 days (table 3).

There is hardly any significant difference in yield if Ca content is 4 p.p.m. (1), 20 p.p.m. (2), or 100 p.p.m. (3). This time the yields are only very little greater on tap water media (4 and 5) than on artificial media (1—3). An addition of Ca to the tap water, making the total Ca concentration as high as 140 p.p.m., does not seem to have any significant effect; Ca concentrations between 4 p.p.m. and 140 p.p.m. seem to be about equally favourable, although the higher concentrations may be a little better.

Experiment 5. For further comparison of growth on artificial and tap water media with approximately equal content of Ca, the series given in table 4 were included in an experiment mainly designed for other purposes (not reported here). Triplicate series were used, 10 ml nutrient medium in 125 ml flasks. pH was adjusted to 5.

In table 4 the relative values are given separately for the asparagine series (no. 2 in per cent of no 1) and the ammonium tartrate series (no. 4 in per cent of no. 3). In this experiment the tap water has no effect apart from that of its Ca content. Evidently the augmentative effect of tap water is subject to some variation.

Experiment 6. The Ca and tap water effects were again compared on both sugar levels previously employed (table 5). Quintuplicate series were used, 10 ml in 125 ml flasks, pH adjusted to 5. The Ca-deficient cultures showed the same deficiency symptoms as before. There was no difference in morphological development between tap water cultures and those on com-

Table 4. Experiment 5. *Growth on tap water media (t.w.) with a natural Ca content of 80 p.p.m., compared with distilled water media (d.w.) with Ca added to the same concentration. Basic medium A 30 (asparagine) or B 30 (ammonium tartrate), +FeZnCuMnMoB.*

No.	Medium	Dry weight of mycelia					pH 4 d.
		2 days mg	3 days		4 days		
			mg	0/o	mg	0/o	
1	A 30, d.w.+Ca	17.9	67.6	100	100.0	100	2.9
2	A 30, t.w.	18.5	74.3	109.6	96.3	96.3	3.0
3	B 30, d.w.+Ca	14.1	56.1	100	77.7	100	3.0
4	B 30, t.w.	13.3	55.4	98.9	81.6	105.0	2.9

plete artificial media. The yields on tap water were, however, in this experiment slightly lower than those on artificial media, in striking contrast to the augmentative effect observed in experiments 1, 2, and 3.

The six experiments reported above were performed in Uppsala. The tap water of this city comes from a subterranean reservoir in a huge moraine west of the town, consisting of calciferous sand and gravel. There is no reason to believe that the mineral composition of the water should be subject to variations that could explain the changes in physiological effect observed in these experiments. It seems more likely that the tap water effect (or rather, the part of the effect which is not due to the Ca content) is caused by some other type of factor, perhaps of organic nature. The experiments where positive effects were found were performed in February and March, while those where no effect, or a slight negative effect was observed were performed in June and July. It might perhaps be a workable hypothesis to assume a seasonal variation in the composition of the organic matter present in the ground water.

After a delay work was resumed in Oslo. The first experiment had to be discarded, as growth of the fungus was poor and irregular, probably because the stock cultures had not been subcultured sufficiently frequently. It was observed, however, that cultures where no Fe or Zn had been added did not display any deficiency symptoms at all. As this was wholly incongruous with previous experiences the chemicals employed were tested with dithizone for heavy metal contaminants (see »Material and Methods»). It was found that the asparagine gave a very strong zinc reaction; obviously this was the cause of the aberrant result. Two other brands of asparagine were tested; one gave a weak copper reaction, while the other gave no reaction with dithizone at pH 5, and was considered to be free of heavy metal contaminants.

Table 5. Experiment 6. Growth on distilled water media without Ca, or with varying additions of Ca (20 and 80 p.p.m.), compared with tap water media (Ca, 80 p.p.m.). Glucose content 20 or 30 g per liter (A 20 or A 30). Added FeZnCuMnMoB.

No.	Medium	Dry weight of mycelia				pH 4½ d.
		3 days		4½ days		
		mg	0/0	mg	0/0	
1	A 20, d.w., no Ca	24.8	60.0	62.3	76.6	5.7
2	» » , Ca, 20 p.p.m.	41.4	100	81.3	100	6.6
3	» » , Ca, 80 p.p.m.	41.7	100.7	82.2	101.1	5.7
4	» , t.w., Ca, ca. 80 p.p.m. ...	36.1	89.6	78.8	97.0	4.6
5	A 30, d.w., no Ca	35.6	61.3	83.5	87.0	3.65
6	» » , Ca, 20 p.p.m.	58.1	100	95.9	100	3.2
7	» » , Ca, 80 p.p.m.	56.2	96.7	98.6	102.8	2.85
8	» , t.w., Ca, ca. 80 p.p.m. ...	47.7	82.1	95.0	99.1	2.9

This asparagine (from Riedel-de Haën), and a glucose brand (Difco) that also proved dithizone-negative, were employed for the following experiments 7 to 11.

A new strain — to be called strain II — was obtained from Centraalbureau voor Schimmelcultures, Baarn, Holland, and was utilized for experiment 7; for the subsequent experiments (8—11) subclones of this or the first strain — strain I — or both, were employed.

The basic medium employed in expts. 7—11 was A 30 (cf. p. 589). Ca was added in an amount of 80 p.p.m., and trace elements as in expt. 4 (p. 594). To secure sufficient carbohydrate supply 15 ml nutrient medium in 300 ml flasks were used, as against 10 ml in 125 ml flasks in experiments 1—6. Both will give a liquid layer of about 3 mm depth.

Experiment 7. The effect of each trace element was investigated by comparing growth on the complete medium »A₇», i.e. A 30 + Ca + Fe + Zn + Cu + Mn + Mo + B (trace elements given from separate stock solutions) with growth on media where *one* of the elements was omitted. Also one series with the basic medium A 30 (called »A₀») was included, and one with Ca only. Quintuplicate series, initial pH 5. The calcium-deficient cultures displayed the familiar deficiency symptoms, sporangiophores being shorter, more or less bent or curled, and sporangia less abundant than on the mycelia in the complete medium. The effect of calcium deficiency upon dry weight yield was, however, less pronounced; relative yield was 90 per cent, against ca. 60 per cent in earlier experiments. The iron-deficient (or insufficient) cultures displayed no definite abnormal morphological features, but appeared to be of a somewhat immature character; the mycelial mats were thinner and looser than those on complete medium, and sporangiophores were less

Table 6. Experiment 7. *Effect of trace element deficiencies upon growth*. Basic medium A 30 (cf. p. 589). Concentrations of trace elements: Ca, 80 p.p.m.; Fe, 0.32 p.p.m.; Zn, 0.44 p.p.m.; Cu, 0.05 p.p.m.; Mn, 0.1 p.p.m.; Mo, 0.1 p.p.m.; B, 0.02 p.p.m.

No.	Medium	Yield (dry weight), 4 days		pH 4 d.
		mg	0/0	
1	A ₇	140.2 ± 3.6	100	3.15
2	A ₇ — Ca	126.3 ± 0.5	90.0	3.6
3	A ₇ — Fe	104.8 ± 10.4	74.9	4.15
4	A ₇ — Zn	44.9 ± 0.8	32.1	4.8
5	A ₇ — Cu	140.6 ± 3.2	100.2	3.1
6	A ₇ — Mn	133.1 ± 4.4	95.1	3.3
7	A ₇ — Mo	148.5 ± 3.7	106.0	3.1
8	A ₇ — B	121.0 ± 8.8	86.3	4.1
9	A ₀	40.3 ± 2.3	28.8	5.0
10	A ₀ + Ca	47.2 ± 5.7	33.7	4.9

developed with only a few young sporangia. Yields were appreciably reduced (compare 3 with 1); difference between individual mycelia was much greater than in the other series, as indicated by the standard error. The zink-deficient mycelia (4) were very small, loose and thin, bearing only very few poorly developed sporangiophores. The appearance of the Fe-deficient and Zn-deficient mycelia was very much different from that of the FeZn-deficient mycelia in experiments 2 and 3 (for description of symptoms see p. 594). Also the effect on yield of absence of Zn alone was very much more pronounced (32 percent) than that of absence of FeZn in earlier experiments (ca. 70 per cent). Absence of Cu (5), Mn (6), or Mo (7) seemed to have no appreciable effect. Of the cultures where B was omitted, one parallel (out of five) was exactly like the control (dry weight 144.0 mg), while the other four were somewhat juvenile in appearance, resembling that described for the Fe-deficient cultures although less pronounced. Yields were somewhat reduced (8), and standard error greater. The possibility that B may have an effect seemed to deserve further attention. — The cultures on minimal solution A₀ were similar to those where Zn alone was omitted; yields were a little lower (compare 9 and 4). Addition of Ca alone (10) had very little effect; these cultures were almost exactly like those on the medium A₇—Zn.

The results obtained in this experiment differ somewhat from those obtained in earlier experiments; the effect of Ca deficiency on yield is less pronounced, the effect of omission of Zn is considerably greater than that observed earlier for omission of FeZn, and an effect of B-deficiency is indicated. As this experiment was carried out with another strain (st. II), a comparison with the old strain (st. I) was desirable.

Table 7. Experiment 8. *Effect of some ion deficiencies upon growth of two different strains, Ia and IIa. Concentrations as in expt. 7, cf. table 6.*

Strain	Medium	Yield (dry weight of mycelia)				E. C. 4 d.	pH 4 d.
		3 days		4 days			
		mg	o/o	mg	o/o		
I a	A ₄ (Ca, Fe, Zn, B) ...	61.4 ± 2.1	100	120.6 ± 3.4	100	40.8	3.6
»	A ₄ — Ca	34.1 ± 1.0	55.5	69.8 ± 1.5	57.9	36.7	4.9
»	A ₄ — FeZn	19.0 ± 1.4	30.9	28.4 ± 2.7	23.5	22.3	4.9
»	A ₄ — B	52.0 ± 3.1	84.7	87.0 ± 6.2	72.1	35.5	4.7
»	A ₇ (A ₄ + Cu, Mn, Mo)			112.3 ± 1.5	94.1	43.8	3.8
II a	A ₄	66.2 ± 1.9	100	133.6 ± 3.1	100	41.6	3.1
»	A ₄ — Ca	54.8 ± 4.7	82.8	109.1 ± 5.2	81.7	40.4	3.8
»	A ₄ — FeZn			24.8 ± 1.4	18.5	19.5	4.7
»	A ₄ — B			124.9 ± 5.1	93.5	42.0	3.2
»	A ₇ (A ₄ + Cu, Mn, Mo)			133.0 ± 1.8	99.6	40.7	3.0

Experiment 8. The following combinations of ions were employed:

- 1: A₀ + Ca + Fe + Zn + B — to be called A₄
- 2: A₄ — Ca
- 3: A₄ — FeZn
- 4: A₄ — B
- 5: A₀ + Ca + Fe + Zn + Cu + Mn + Mo + B = A₇

The two strains were compared; as homogeneity was thought to be a little unsatisfactory in the foregoing experiment, clones were isolated from either strain through inoculation of agar slants with single spore mycelia. The spores from two of these clone cultures (to be called Ia and IIa) were sown in the usual way (cf. Material and Methods). Two harvests (after three and four days) were taken of most series; of some series however only one harvest (four days) was taken. Quintuplicate series were generally employed. To obtain a comparison of homogeneity of the two strains ten parallels were harvested after three days of both strains on the medium A₄.

Culture conditions were the usual ones. Economic coefficients (E.C.) were computed from the yields of the four-day harvest for the different media employed.

The general morphological characters of the series were identical for both strains. The Ca-deficiency symptoms were the same as before. The cultures on media lacking Fe and Zn had an appearance exactly similar to that of the Zn-deficient cultures in the foregoing experiment (expt. 7), but entirely different from that observed for Fe-Zn-deficient media in earlier experiments. The yield is very low — ca. 20 per cent, as against ca. 70 per cent in experiments 2 and 3. Evidently some contamination of the chemicals

Table 8. Experiment 9. *Effect of calcium and boron deficiency upon growth of two different strains, Ib and Iib. Concentrations as in experiment 7, cf. table 6.*

Strain	Medium	Yield (dry weight of mycelia)				E. C. 4 d.	4 d pH
		3 days		4 days			
		mg	0/o	mg	0/o		
I b	A ₄ (Ca, Fe, Zn, B) ...	47.4 ± 1.4	100	91.5 ± 2.1	100	35.5	4.4
»	A ₄ — Ca	29.0 ± 1.6	61.2	67.8 ± 2.4	74.1	38.9	4.8
»	A ₄ — B	40.8 ± 1.6	90.3	88.1 ± 3.6	96.3	32.8	4.4
II b	A ₄ (Ca, Fe, Zn, B) ...	64.3 ± 3.1	100	128.4 ± 3.2	100	41.5	3.2
»	A ₄ — Ca	48.1 ± 3.2	74.8	97.7 ± 4.1	76.1	40.0	3.9
»	A ₄ — B	55.7 ± 2.4	86.6	123.0 ± 2.7	95.8	39.8	3.2

employed for the last named experiments, in all probability of the asparagine, is the cause of the relatively great yield. — The effect of boron deficiency seems to be confirmed for strain Ia; at least some of the mycelia were a little immature in appearance, and yield was somewhat lower than that of the control. Strain Iia, however, does not seem to be appreciably influenced by absence of boron. — While the effect of Ca-deficiency on dry weight yield is very pronounced for strain Ia, it is not so great for strain Iia. The relative yield for strain Ia (56—57 %) is in good agreement with results obtained in earlier experiments with strain I (ca. 60 %), and for st. Iia (81.7 %) with that obtained in expt. 7 (90 %). A difference is indicated between st. I and st. II (and their substrains) in the degree of quantitative response to Ca deficiency. The qualitative response, i.e. abnormality of morphological development, is, however, identical. An addition of Cu, Mn and Mo to the A₄ medium — giving A₇ — does not seem to have any positive effect.

Strain Ia gave smaller yields than strain Iia, and was not quite so healthy in appearance.

Experiment 9. A comparison was made between strain Ib and strain Iib, parallel clones to those employed in expt. 8, on the following media: A₄ (A₀+Ca, Fe, Zn, and B), A₄—Ca, and A₄—B. Ten parallels were made up of each series and strain. Mycelia were harvested after 3 and 4 days. Culture conditions were as usual.

Strain Ib proved to be much weaker than Iib, and gave a somewhat unhealthy appearance. There is no significant difference between these two strains in response to Ca deficiency. The boron-deficient cultures were only little different in appearance from the controls, although the sporangiophores of some of the cultures of strain Iib were a little less developed, rendering a slightly immature character to the mycelia. The yields of the boron-deficient cultures were not much different from those of the control series.

Table 9. Experiment 10. *Effect of various ion deficiencies, and of addition of some natural ion mixtures, viz. sea water, and tap water from Oslo and Uppsala. Concentrations as in expt. 7, cf. table 6. For further explanation see text.*

No.	Medium	Yield, 4 days		E.C.	pH 4 d.
		mg	o/o		
1	A ₇	139.8 ± 3.2	100	41.1	2.8
2	A ₇ — Ca	120.0 ± 3.0	85.7	36.1	3.4
3	A ₇ — Fe	106.2 ± 5.6	75.9	37.3	3.7
4	A ₇ — Zn	27.8 ± 1.6	20.0	17.5	4.5
5	A ₇ — Cu	123.5 ± 6.1	88.2	39.7	3.3
6	A ₇ — Mn	119.0 ± 4.3	85.0	38.0	3.3
7	A ₇ — Mo	136.2 ± 2.5	97.3	40.7	3.0
8	A ₇ — B	144.4 ± 2.1	103.1	40.6	2.8
9	A ₄	136.4 ± 2.2	97.4	39.8	2.9
10	A ₄ — B	135.6 ± 1.7	96.9	40.1	2.8
11	A ₄ + s.w.	138.0 ± 1.4	98.6	36.5	2.7
12	A ₄ + NaCl	136.0 ± 4.1	97.1	39.2	2.9
13	A ₄ — Ca, twU	138.9 ± 4.4	99.2	38.3	2.7
14	A ₄ , twO	144.0 ± 1.6	102.9	40.5	2.8

Experiment 10. A comparison was made between growth on media with additions of some natural sources of ions, and that on synthetic media. The following combinations were employed:

- 1: A₇ = A₀ + Ca + Fe + Zn + Cu + Mn + Mo + B
- 2—8: A₇ — one of the trace elements
- 9: A₄ = A₀ + Ca + Fe + Zn + B
- 10: A₄ — B
- 11: A₄ + s.w.; sea water, stored in the dark for several months, was added in amounts of 40 ml per liter
- 12: A₄ + NaCl, in a quantity to make the NaCl concentration equal to that of solution 11
- 13: A₄ — Ca, t.w. U.; the solution was made up from tap water from Uppsala instead of distilled water. As the natural Ca content of this water is approximately equal to that of the complete solution, addition of Ca was omitted.
- 14: A₄, t.w. O.; the solution was made up from tap water from Oslo's water-works; this water is low in Ca content, so that Ca was added in the usual amount.

Strain IIa was used. Ten or eight parallels were made up of each series; culture conditions as usual. Mycelia were harvested after 4 days. The deficiency symptoms of the cultures where Ca, Fe, or Zn were lacking were the same as before; also the relative yields were in good agreement with those obtained in earlier experiments. Some of the mycelia of series 5 and 6, lacking

Cu and Mn respectively, appeared slightly immature, and yields were somewhat lower than that of the control. Omission of B had no effect in this experiment (cf. 8 with 1, and 10 with 9). The slight deficiency effect in series 5 and 6 (—Cu and —Mn) was not confirmed by series 9 and 10; the omission of the complex CuMnMo, or of CuMnMoB, had no appreciable effect on yield. The possible explanation for these incongruities will be discussed in connection with the next experiment. An addition of sea water (11) to a medium containing Ca, Fe, Zn, and B had no effect. NaCl in a concentration corresponding to that caused by the addition of sea water was not harmful (12). Media made up of tap water (13 and 14) did not give yields that were appreciably greater than that on the corresponding distilled water medium (9). The tap water of Oslo is taken from lakes in woodland district where the ground consists of igneous rocks, poor in calcium; it is presumably entirely different in ion content from the highly calciferous tap water of Uppsala (cf. p. 596).

Experiment 11. In this experiment Ga was included among the trace elements. No Ga compound could be obtained in Oslo, but a small sample of $\text{Ga}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}$ was placed at my disposal through the courtesy of the Chemical Research Laboratories, Teddington, Middlesex, England. From a stock solution this compound was added to the nutrient medium to give a concentration of 0.1 p.p.m. of Ga, the same concentration as that used for Mn and Mo. The following combinations of ions were employed:

- 1: $A_8 = A_0 + \text{Ca} + \text{Fe} + \text{Zn} + \text{Cu} + \text{Mn} + \text{Mo} + \text{B} + \text{Ga}$
- 2: $A_8 - \text{Ca}$
- 3: $A_3 = A_0 + \text{Ca} + \text{Fe} + \text{Zn}$
- 4—6: $A_3 - \text{Ca}, \text{Fe or Zn}$
- 7—11: $A_3 + \text{one of the elements Cu, Mn, Mo, B, or Ga.}$
- 12: $A_0 = \text{basic solution with no trace elements added.}$

Strain IIb was employed. Five parallels were made up of series 1 and 2, while eight parallels were made up of the other series. Mycelia were harvested after 4 days. Culture conditions were the usual ones.

The effect of omission of Ca, Fe, or Zn was essentially the same as in earlier experiments. Zn is by far the most important of these elements; omission of this element alone had an effect that was almost identical to that of omission of all three elements (compare 6 and 12). Zn deficiency influences the economic coefficient as well as dry weight yield and morphological development. Addition of the other trace elements employed, including Ga, to a solution containing Ca, Fe, and Zn had no augmentative effect on yield or morphological development; whether the elements Cu, Mn, Mo, B, and

Table 10. Experiment 11. *Effect of Ca, Fe, and Zn deficiency, and of addition of Cu, Mn, Mo, B, and Ga, singly or in combination. Concentrations as in expt. 7, table 6. Ga, 0.1 p.p.m.*

No.	Medium	Yield, 4 days		E. C.	pH 4. d.
		mg	%		
1	A ₈	142.4 ± 4.1	100	41.3	2.9
2	A ₈ — Ca	116.9 ± 2.6	82.1	38.7	3.3
3	A ₈ (Ca, Fe, Zn)	138.6 ± 4.6	97.4	39.4	2.9
4	A ₈ — Ca	115.4 ± 3.4	81.1	38.1	3.5
5	A ₈ — Fe	115.8 ± 2.5	81.3	39.5	3.4
6	A ₈ — Zn	23.7 ± 1.5	16.6	16.5	4.7
7	A ₈ + Cu	138.2 ± 3.1	97.1	40.8	2.9
8	A ₈ + Mn	141.9 ± 2.3	99.6	41.3	2.9
9	A ₈ + Mo	138.4 ± 3.8	97.2	41.1	2.9
10	A ₈ + B	135.1 ± 2.4	94.8	40.1	2.9
11	A ₈ + Ga	140.9 ± 2.8	98.9	41.8	2.9
12	A ₀	20.2 ± 1.3	14.1	16.1	5.0

Ga were added singly (7—11) or in combination (1) they seemed to be without any appreciable effect.

In earlier experiments the omission of one of these elements was sometimes observed to have a slight negative effect; development seemed to be retarded so that the mycelia would appear more or less immature after four days growth, and yield would be somewhat reduced (effect of B in expts 7 and 8, of Cu and Mn in expt. 10). However, the effect was somewhat inconsistent, and, moreover, no reduction was observed when *all* of these elements were omitted (expts. 10 and 11), or when only one of them was added to a solution containing the essential elements Ca, Fe and Zn (expt. 11). Evidently Cu, Mn, Mo, B and Ga do not need to be added to secure optimum growth and development. The reduction in yield sometimes observed when one of them is omitted (B, Cu or Mn) while the others are added, might perhaps be ascribed to an antagonistic effect. The evidence is, however, wholly insufficient for any conclusions to be drawn.

Discussion

The experiments reported in this paper have demonstrated that Ca, Fe, and Zn are necessary for normal development and optimum growth of *Phycomyces blakesleeanus*. Omission of *Ca* will reduce dry weight yield to a varying degree (55—90 per cent of yield on the complete medium). A difference in quantitative response to Ca deficiency between the two strains employed seems to be indicated; while strain I would give relative yields from 55 to 77 per cent, strain II would give from 75 to 90 per cent. The morphological abnormalities called forth by Ca deficiency were essentially

the same for both strains. Omission of *Fe* had almost identical effect in three experiments; yields were reduced to 74.9, 75.9 and 81.6 per cent respectively, and development seemed to be retarded. The effect of *Zn* deficiency was very pronounced — yields being 32.1, 20.0 and 16.6 per cent in three different experiments; a dithizone test had proved the macronutrients to be free from heavy metal contaminants. Omission of both *Fe* and *Zn* gave an effect almost identical to that of omission of *Zn* alone in one experiment where dithizone-negative macronutrients were employed; also omission of all the three essential elements *Ca*, *Fe* and *Zn* would give yields and morphological characters very similar to those obtained when *Zn* alone was omitted. Evidently *Zn* plays a very important role in metabolism. The reduction of economic coefficient when *Zn* is omitted also serves to demonstrate the essential character of this element. When in two earlier experiments (expts. 2 and 3) omission of *Fe*+*Zn* did not call forth a reduction in yield of the same magnitude this is no doubt due to the employment of macronutrients of insufficient purity; the chemicals were not tested with dithizone on this occasion. The quantitative effect was, however, considerable also in these experiments: yields were from 67 to 80 per cent of controls, and the morphological abnormalities were very pronounced.

The other trace elements tested, viz. *Cu*, *Mn*, *Mo*, *B*, and *Ga*, were not proved to have an effect on growth or development (cf. p. 603). Nor did natural mixtures of ions, such as tap water and sea water, have any additional augmentative effect when added to a complete artificial medium.

The conclusion that can be drawn from the experiments reported in this paper is that addition of *Ca*, *Fe* and *Zn* is necessary, and sufficient, to secure maximum growth of *Phycomyces blakesleeanus*.

It should be emphasized, however, that this will hold true only for the conditions employed in these experiments, viz.: 1: a basal medium containing glucose, asparagine, monopotassium phosphate and magnesium sulfate, 2: Pyrex glassware, 3: distilled water redistilled in Pyrex, 4: C.P. chemicals, not especially purified, but proved to give no colour reaction to dithizone at pH 5.

As mentioned on p. 596 no zinc deficiency effect could be observed when another brand of asparagine was used, but this asparagine gave a positive *Zn* reaction when tested with dithizone; also the difference between experiments 2 and 3, and later experiments in degree of response to omission of *Fe*+*Zn* is probably due to contaminants in the asparagine. Other investigators report to have observed no effect of *Zn* or of a mixture of ions including *Zn* (Schopfer 1934, Robbins and Kavanagh 1941, Robbins and Hervey 1944, Thren 1941), or else a much less pronounced effect (Schopfer 1938, Hurni 1945). Leonian and Lilly (1940 b) observed a reduction in yield to about

50 % of the control when Zn alone was omitted. In the present investigation a relative yield as low as 16.6 per cent was observed in a Zn-deficient medium (experiment 11). The differences in degree of response to Zn deficiency between the various investigations may be due not only to differences in the purity of chemicals, but also to the type of glass utilized. Javillier (1914) observed that *Aspergillus niger* displayed no deficiency symptoms when cultivated in Jena glass vessels on a medium without Zn; his figures are cited:

	En verre de Boheme	En verre d'Iena	En vase de quartz
Cultures témoins	0,352	1,861	0,291
Cultures avec zinc	1,780	1,736	1,624
Même expérience avec une autre race de <i>Sterigmatocystis nigra</i> :			
Cultures témoins	0,308	1,365	0,219
Cultures avec zinc	1,823	1,762	1,645

As Jena glass vessels are very often used for culture experiments (for instance in several investigations by Schopfer), this may be the reason why omission of Zn is not observed to have any effect.

Evidently the effects of Ca, Fe and Zn reported in this paper can not be expected to be observed in all cases if ionic composition of the medium is less strictly controlled.

On the other hand, if special purification precautions were taken, further requirements for trace elements might perhaps be revealed. Steinberg (1950 c, and several earlier contributions) has demonstrated that different purification procedures, or combinations of procedures, may give quite different percentage yields on omission of trace elements. He states (l.c. p. 115): »The writer has previously estimated that a purity of one part per billion would be necessary in order to identify all the micronutrients necessary for microorganisms. Present levels of purity are probably ten times lower. These impurities presumably affect the results obtainable with the known essential elements, and may therefore lead to erroneous results and interpretations.»

No attempt was made in the present investigation to analyse mineral requirements under »absolute» control of ionic composition of the medium (as far as this ideal can be achieved). The purpose was to find out which trace elements may affect growth under conditions suited for further investigations on the physiology of *Phycomyces*, and for vitamin assays. It is hoped that a »relative» degree of control has been achieved that may be considered reproducible and defined. At pH 5 the dithizone test is very sensitive for Cu, Hg and Zn (Fischer 1937, Sandell 1944). Other heavy metals that may be biologically active (Fe, Mn, etc.) can not be detected in acid solution. (Several additional elements will give colour reactions in alkaline milieu).

It may be considered, however, that if the macronutrients and the distilled water employed for the nutrient medium are free from traces of the very common contaminants Cu and Zn, their general degree of purity will be sufficient for physiological experiments. Also the dithizone test has the advantage of being easy and quick to carry out.

As a conclusion of this investigation the following nutrient solution is recommended for *Phycomyces*:

Glucose	30 g	Fe+++	0.32 mg
Asparagine	3.5 g	Zn++	0.44 mg
KH ₂ PO ₄	1 g	Thiamine	50 µg
MgSO ₄ · 7 H ₂ O	0.5 g	Redist. water	1 liter
Ca++	80 mg		

This medium was found to secure maximum growth, and if an addition of other organic compounds or natural substances is found to have an augmentative effect this probably will not be due to their mineral content. However, if further security concerning completeness of mineral content is considered desirable, an addition of sea water (stored for a long period in the dark) may be recommended.

It should be emphasized, though, that this medium has one serious disadvantage — it is poorly buffered in the pH range 5—3. A considerable acidification will take place during the culture period, bringing pH considerably below the range where optimum growth was found to occur (ca. 4—4.5). Experiments are in progress to overcome this difficulty.

Summary

1. Calcium, iron and zinc were demonstrated to be necessary for optimum growth and normal development of *Phycomyces blakesleeanus*.

2. Further addition of copper, manganese, molybdenum, boron and gallium had no consistent augmentative effect; their addition is not considered to be necessary under the culture conditions employed.

3. Additions of natural mixtures of ions, viz. sea water and two different types of tap water, were not found to have any consistent augmentative effect. A positive effect of tap water observed in some experiments is ascribed to other factors than the mineral content, probably of organic nature.

4. It is emphasized that the effects observed hold true only under the culture conditions employed, viz.: glucose and asparagine as carbon and nitrogen sources, Pyrex glassware, redistilled water (Pyrex still), and C.P. chemicals, not especially purified, but proven free of heavy metal contaminants by testing with dithizone at pH 5.

5. A nutrient medium is recommended that will secure maximum growth and normal development of *Phycomyces blakesleeanus*.

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References

- Bang, H. O.: B₁-Vitaminstudier ved Hjælp av *Phycomyces*metoden. — Diss. Vilhelm Priors Forlag, København 1944.
- Basu, C. N.: Significance of calcium in the fruiting of *Chaetomium* species, particularly *Chaetomium globosum*. — Jour. Gen. Microbiol. 5: 231. 1951. [Ref.: Biol. Abstr. 25 (10) No. 31687. 1951.]
- Burgeff, H.: Pflanzliche Avitaminose und ihre Behebung durch Vitaminzufuhr. — Ber. dtsch. bot. Ges. 52: 384. 1934.
- Burkholder, P. R. & McVeigh, I.: Growth of *Phycomyces blakesleeanus* in relation to varied environmental conditions. — Am. J. Bot. 27: 634. 1940.
- Davis, A. R., Marloth, R. H. & Bishop, C. J.: The inorganic nutrition of fungi I. The relation of calcium and boron to growth and spore formation. — Phytopathology 18: 949. 1928.
- Feigl, F.: Spot Tests. — 3rd ed. Elsevier Co., N.Y. 1947.
- Fischer, H.: Dithizon als Reagens in der qualitativen und quantitativen Mikroanalyse. — Angew. Ch. 47: 685. 1934.
- Dithizonverfahren in der Chemischen Analyse. — Angew. Ch. 50: 919. 1937.
- Foster, J. W.: Chemical Activities of Fungi. — Academic Press. N.Y. 1949.
- Haag, E. & Dalphin, C.: Un nouveau principe de dosage de la vitamine B₁ à l'aide du *Phycomyces blakesleeanus*. — C. r. soc. phys. hist. nat. Genève 61: 241. 1944.
- Hurni, H.: Zusatzwachstumsfaktoren für *Phycomyces blakesleeanus*. — Z. Vitaminforsch. 16: 69. 1945.
- Jarvis, F. G. & Johnson, M. J.: The role of constituents of synthetic media for penicillin production. — J. Am. Chem. Soc. 69: 3010. 1947.
- The mineral nutrition of *Penicillium chrysogenum*. — J. Bact. 59: 51. 1950.
- Javillier, M.: Une cause d'erreur dans l'étude de l'action biologique des éléments chimiques: la présence de traces de zinc dans le verre. — C. r. Acad. sci. 158: 140. 1914.
- Kocher, V.: Bestimmung des Gesamt-Aneuringehaltes von Lebensmitteln mit Hilfe des *Phycomycestestes* nach einem geänderten Verfahren (Kompensationstest). — Z. Vitaminforsch. 15: 83. 1944.
- Lehmann, J. & Nielsen, H. E.: B₁-Vitaminbestimmung im Blut nach Schopfer's *Phycomyces*methode. — Acta Med. Scand. Suppl. 123: 374. 1941.

- Leonian, L. H. & Lilly, V. G.: Auxithals synthesized by some filamentous fungi. — *Plant Physiol.* 15: 515. 1940.
- — Factors influencing the growth of some thiamine requiring fungi. — *Am. J. Bot.* 27: 18. 1940 b.
- — Factors affecting zygospore formation in *Phycomyces blakesleeanus*. — *Am. J. Bot.* 27: 670. 1940 c.
- Lilly, V. G. & Barnett, H. L.: *Physiology of the Fungi*. — 1st ed. McGraw Hill Book Co., N.Y. 1951.
- Lindeberg, G.: Ueber die Physiologie ligninabbauender Hymenomyceten. — *Symb. Bot. Upsal.* VIII No. 2. 1944.
- Lohmann, K. & Cheng, C. T.: Ueber den Einfluss von Magnesium- and Mangansalzen auf das Wachstum von *Phycomyces*. — *Naturwiss.* 28: 172. 1940.
- Mann, M. L.: Calcium and magnesium requirements of *Aspergillus niger*. — *Bull. Torrey Bot. Cl.* 59: 443. 1932.
- Meiklejohn, A. P.: The estimation of vitamin B₁ in blood by a modification of Schopfer's test. — *Biochem. J.* 31: 1441. 1937.
- Melin, E.: Der Einfluss von Waldstreuextrakten auf das Wachstum von Bodenpilzen, mit besonderer Berücksichtigung der Wurzelpilze von Bäumen. — *Symb. Bot. Upsal.* VIII No. 3. 1946.
- Mosher, W., Saunders, D., Kingery, L. & Williams, R.: Nutritional requirement of the pathogenic mold *Trichophyton interdigitale*. — *Plant Physiol.* 11: 795. 1936.
- Mulder, E. G.: Importance of molybdenum in the nitrogen metabolism of microorganisms and higher plants. — *Plant and Soil* 1: 94. 1948.
- Norkrans, B.: Studies in growth and cellulolytic enzymes of *Tricholoma*. — *Symb. Bot. Upsal.* XI No. 1. 1950.
- Omvik, Aa.: Experiments on manganese, zinc and calcium as constituents of nutrition solutions for fungi. — *Univ. Bergen Årbok* 1951. nat. vit. rekke nr. 8. 1951.
- Perlman, D.: Effect of minor elements on the physiology of fungi. — *Bot. Rev.* 15: 195. 1949.
- Rippel, A. & Stoss, H.: Ist Calcium ein für Mikroorganismen notwendiges Element? — *Arch. Mikrobiol.* 3: 492. 1932.
- Robbins, W. J. & Hervey, A.: Response of *Pythiomorpha gonapodyides* to manganese. — *Bull. Torrey Bot. Cl.* 71: 258. 1944.
- & Kavanagh, F.: Vitamin B₁ or its intermediates and growth of certain fungi. — *Am. J. Bot.* 25: 229. 1938.
- — Thiazole effect on *Phycomyces*. — *Proc. Nat. Acad. Sci.* 27: 423. 1941.
- & Kavanagh, V.: Vitamin deficiencies of the filamentous fungi. — *Bot. Rev.* 8: 411. 1942.
- Schmitt, M. B.: Factor Z₂ and gametic reproduction by *Phycomyces*. — *Am. J. Bot.* 32: 320. 1945.
- Sandell, E. B.: *Colorimetric Determination of Traces of Metals*. — N.Y. 1944.
- Schopfer, W. H.: Les vitamines cristallisées B comme hormones de croissance chez un microorganisme (*Phycomyces*). — *Arch. Mikrobiol.* 5: 511. 1934.
- Recherches sur l'action des produits animaux sur le développement d'un microorganisme (*Phycomyces*). L'action du lait. — *Arch. Mikrobiol.* 8: 231. 1937.
- Vitamine und Wachstumsfaktoren bei den Mikroorganismen, mit besonderer Berücksichtigung des Vitamins B₁. — *Erg. Biol.* 16: 1. 1939.
- Le problème de la spécificité d'action des vitamines. Etudes de quelques analogues de l'aneurine (vitamine B₁). — *C. r. soc. phys. hist. nat. Genève* 58: 58. 1941.

- La biosynthèse de la biotine par *Phycomyces blakesleeanus*. Nouvelle facteur de croissance pour la levure. — *Z. Vitaminforsch.* 14: 42. 1943.
- Plants and Vitamins. — *Chronica Botanica Book. Co.* 1943, repr. 1949.
- Les tests microbiologiques pour la détermination des vitamines. — *Experientia* 1: 183, 1: 219. 1945.
- & Utiger, H.: L'eau de mer, source de catalyseurs minéraux pour la culture d'un microorganisme. — *C. r. soc. phys. hist. nat. Genève.* 58: 135. 1941.
- Sinclair, H. M.: The estimation of vitamin B₁ in blood. — *Biochem. J.* 32: 2185. 1938.
- The estimation of vitamin B₁ in blood II. A further modification of Meiklejohn's method. — *Bioch. J.* 33: 2027. 1939.
- Snell, F. D. & C. T. Snell: *Colorimetric Methods of Analysis.* — D. Van Nostrand Co., N.Y. 1936.
- Steinberg, R. A.: The essentially of gallium to growth and reproduction of *Aspergillus niger*. — *J. Agr. Res.* 57: 569. 1938.
- Specificity of potassium and magnesium for growth of *Aspergillus niger*. — *Am. J. Bot.* 33: 210. 1946.
- Essentiality of calcium in the nutrition of fungi. — *Science* 107: 423. 1948.
- Growth on synthetic nutrient solution of some fungi pathogenic to tobacco. — *Am. J. Bot.* 37: 711. 1950.
- Growth of fungi in synthetic nutrient solutions II. — *Bot. Rev.* 16: 208. 1950.
- Trace element impurities in nutrient solutions for fungi. — *Arch. Bioch.* 28: 111. 1950.
- Stiles, W.: *Trace elements in Plants and Animals.* — McMillan Co. N.Y. 1946.
- Stout, P. R. & Arnon, D. I.: Experimental methods for the study of copper, manganese and zinc in the nutrition of higher plants. — *Am. J. Bot.* 26: 144. 1939.
- Thren, R.: Kritische Versuche zum biologischen Pilztest (*Phycomyces*-Wachstumstest) auf Vitamin B₁. — *Vitamine u. Hormone* 1: 100. 1941.
- Treschow, C.: Nutrition of the cultivated mushroom. — Diss. Ejnar Munksgaard, Copenh. 1944.
- Utiger, H.: Vitamin B₁-Untersuchungen in Gemüsen mittels des *Phycomyces*-Wachstumstestes. — *Z. Vit. f.* 18: 35. 1946.

Distribution of Ca^{45} and P^{32} in Protein Fractions as a Result of Absorption by Potato Tuber Slices

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It has been conclusively shown by several investigators that ion accumulation consists of two parts — a non-metabolic adsorption on the cell surface and a metabolically controlled transfer of the adsorbed ions into the cell (12, 14). The substance or structure responsible for the adsorption is not known, though suggestions have been made (3, 8, 10). Since the cell surface consists of lipids and proteins (5), it would seem logical to suspect the proteins, particularly in view of their amphoteric nature. The purpose of the following investigation was to find out whether any protein fraction (or, fractions) shows an accumulation of the ions absorbed by living tissues.

Methods and Materials

Ca^{45} was obtained in four-millicurie lots from the Oak Ridge National Laboratory. It was supplied as $\text{Ca}^{45}\text{Cl}_2$ in solution with inert calcium as a carrier. Specific activity of the different lots varied as did the amounts of carrier. Necessary dilutions were estimated from the specific activity after correction was made for decay. Total calcium was calculated on the basis of the ratio of radiocalcium to total calcium of the original solution. Specific activity of the fractions was calculated on the basis of a millicurie equalling 3.7×10^7 disintegrations/sec. The fractions were stored in a cold room at 2°C . until needed. The potatoes were peeled, washed, and sliced with a potato slicer into one to two millimeter slices, which were washed in several changes of distilled water, and then placed in 400 ml. of the radioisotope solution in

the absorption cells. The absorption cell consisted of a glass specimen jar covered with a brass lid fitted with a tube extending about an inch on both sides. A sintered glass pyrex gas scrubber was attached to the tube below the lid by means of a rubber tube. The tube above the lid was connected to the aerator. The calculated activity was held constant at about 0.04 microcuries per milliliter in most of the experiments. In preliminary tests the concentration was varied using solutions of 0.004, and 0.04, and 4.0 microcuries per milliliter. The solutions were forcibly aerated throughout the absorption period (usually one hour). pH was determined by a line-operated Macbeth pH meter. In earlier experiments with Ca^{45} , the original pH of the diluted solution was 2.8, but during a one-hour absorption period it rose to 4.85. This low pH failed to produce any visible injury, but is not high enough for optimum absorption (2). The pH of subsequent experiments was buffered with a succinic acid — KOH buffer at pH 6.0. This pH was not appreciably altered during absorption, and the amount taken up by the slices was greater than at the lower pH.

In those experiments utilizing prewashed tissues, the slices were washed for twenty-four hours in running tap water then washed for thirty minutes in several changes of distilled water, and finally transferred to the absorption cells.

When the absorption period was completed, the aeration was stopped, and the tissue slices were removed and washed in distilled water. They were blotted dry with paper toweling, placed between fresh layers of toweling, and frozen quickly with liquid air. The frozen material was buried in »activated alumina» (Al_2O_3) in a vacuum desiccator. The desiccator was evacuated to less than 0.5 mm. Hg with a »Hi-Vac» pump. The freeze-dried slices were removed when dry (after 48 hours), and ground in a Wiley mill using the sixty mesh screen. The resulting powder was stored over a desiccant until used for extraction.

The protein extraction and fractionation techniques have been described elsewhere (7). The freeze-dried protein fractions or a portion of each were wet ashed by the nitric-perchloric acid method. The resulting residue was taken up in three ml. of 3 N HCl and one ml. transferred to each of three one-inch pyrex planchets. In the case of solutions (e.g., dialysates), one ml. was transferred to the planchets directly without ashing. All were dried under a heat lamp and cooled prior to counting.

The dried samples were counted with a Berkeley Model 1000-B.G.M. scaler using a thin mica end-window G.M. tube of 1.9 mg/cm^2 in a Tracerlab model 9 B, shielded, manual, counting chamber. Geometry averaged about 10 per cent for Ca^{45} , 48 per cent for P^{32} as calculated by using a Co^{60}

standard source. Reproducibility was within 1—5 per cent, except for some samples which counted very near background.

Three counts were taken on each of the three replicate samples and averaged. This result was corrected for background, and the activity for that fraction was calculated. Specific activity was calculated from the resulting data.

Results

a) *Uptake of Ca^{45} by tuber slices.*

Preliminary tests were made to determine the optimum conditions. As is to be expected, and as has been found by others, uptake increased with the activity of the external solution (Figure 1). This was true of the slices as a whole as well as of the proteins extracted from them. The absorption time seemed to make little difference between times of ten minutes and four hours (Figure 2). This would seem to indicate that only the adsorption or exchange phase of uptake was involved, which is to be expected in such short periods of time, particularly since the slices were not prewashed (13).

Further evidence that adsorption was mainly involved is obtained from the similarity of the above results at 25° C. with uptake at 2° C. (Figure 3). But the total uptake was about $10 \times$ as much at the higher temperature, yielding a Q_{10} of nearly 3. This high value cannot be explained by simple adsorption or exchange. It may conceivably indicate that respiration increases the number of loci available for adsorption at the protoplast surface.

b) *Uptake of Ca^{45} by proteins (fractionation method 1).*

The above results show that a very small but significant fraction of the absorbed Ca^{45} was in the proteins. Preliminary fractionation seemed to indicate that most of the activity was in the soluble fraction (Figure 2), though the results were variable (Figure 3). Further fractionation was attempted to determine whether any specific protein components were involved. But when an attempt was made to construct a balance sheet, it was found that each fractionation step caused a loss in activity (Table 1). Such large losses always occurred, though the proportions were not constant. Sometimes only 5—10 % of the original activity was recovered in the daughter fractions. Since each fractionation step involved a separate dialysis, it was suspected that the loss occurred during this process. Tests of the dialysate confirmed this (thus in Experiment II dialysates ranged from 6090 to 40850 c/m total activity). The loss during dialysis is to be expected, partly due to simple leaching, partly because many proteins are denatured in salt-free water. This may not be the only cause of the loss in activity. Harman (6)

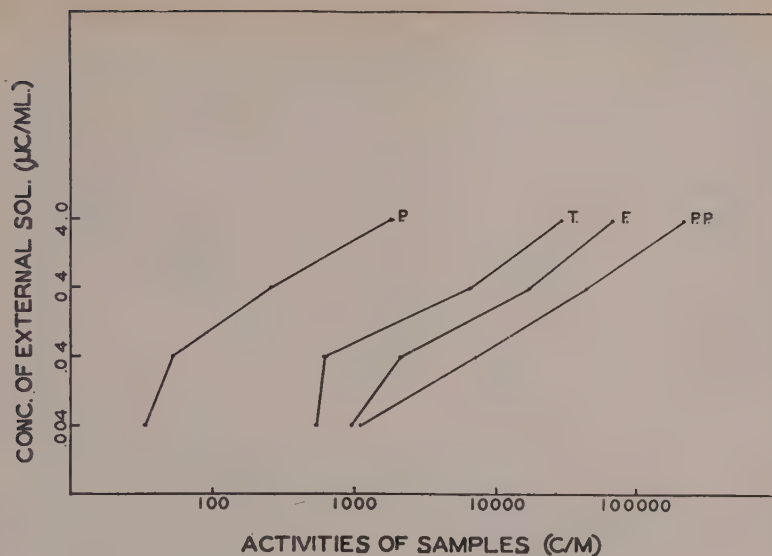


Figure 1. Absorption of Ca^{45} by tuber slices from different concentrations during 24 hours at 25°C .

P=proteins

F=Filtrate (after salting out proteins)

T=Tissue residue

P.P.=Potato powder

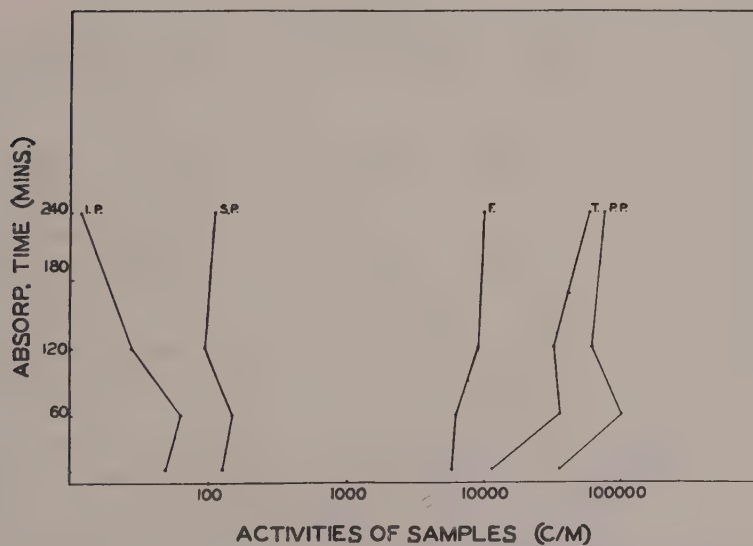


Figure 2. Absorption of Ca^{45} by tuber slices during different periods of time. Activity of solution $0.04 \mu\text{C}/\text{ml}$, temp. 25°C . I.P.=Insoluble Protein; S.P.=Soluble Protein.

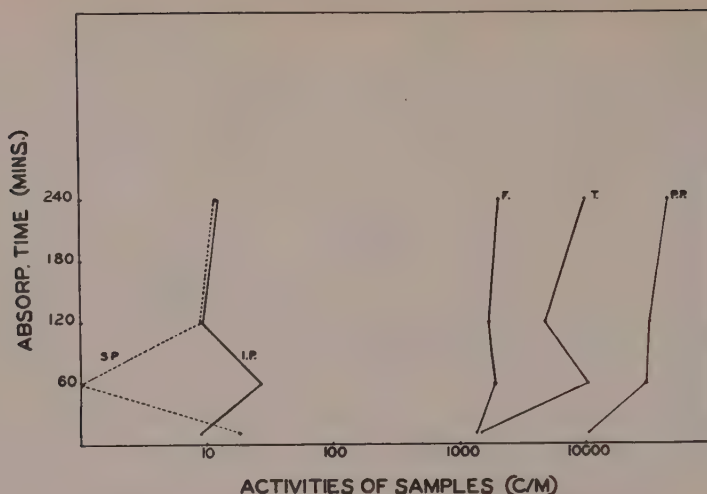


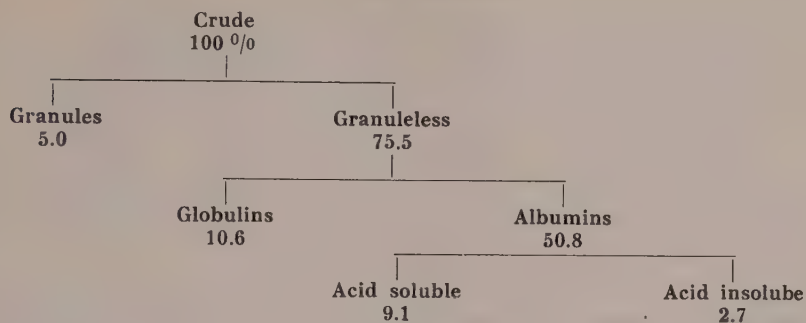
Figure 3. Absorption of Ca^{45} by tuber slices during different periods of time. Activity of solution $0.04 \mu\text{c/ml}$, temp. 2°C .

has shown that $(\text{NH}_4)_2\text{SO}_4$ saturation causes large losses of protein from mitochondria, and this may conceivably be the reason for the low activity of the granules (see method 2).

c) *Uptake of Ca^{45} by proteins* (fractionation method 2).

By use of this method, dialysis is avoided except in the case of the last two fractions (7), consequently losses of activity due to dialysis do not occur in the first three fractions. Table 2 shows that this results in a far greater recovery of activity in the proteins.

The results with fractionation method 2 are particularly interesting with respect to the distribution of the activity among the protein fractions. The order was very different from that obtained by method 1. Method 2 undoubtedly gives a picture closer to the normal in the living cells, since it avoids losses due to dialysis and $(\text{NH}_4)_2\text{SO}_4$ precipitation in three of the fractions. The microsomes had some $20 \times$ the activity of the acid-soluble albumins and over $80 \times$ that of the globulins (Table 3, exp. 12 & 13). That these differences are not simply due to dialysis is shown by the fact that the undialyzed granules usually had much higher activities than the undialyzed acid insoluble proteins. The small activity of the dialyzed albumins, on the other hand, was significantly greater than the negligible activity of the dialyzed globulins. Though the first three fractions comprise only 35–40 per cent of the total proteins (7), they account for 90–95 per cent of the total protein activity.

Table 1. *Effect of Progressive Fractionation on Percent of Total Protein Activity Recovered.*Table 2. *Percent of Activity of Potato Slices Recovered in the Proteins.*

Fractionation Method 1		Fractionation Method 2	
Experiment Number	% of Activity in Proteins	Experiment Number	% of Activity in Proteins
9	2.30	12	8.40
10	1.38	13	13.71
11	0.38	14	16.33

Table 3. *Activities of protein fractions after absorption from 0.032 μ C Ca^{45} /ml. at 25° C. (pH 6.5—6.8). Absorption time 1 hour in experiments 12 and 13, 24 hours in experiment 14. Prewashed 24 hours in experiments 13 and 14. — Counts/minute/mg.*

Fraction	Experiment 12	Experiment 13	Experiment 14
Mitochondria	11.4	14.1	173.6
Microsomes	25.4	13.9	151.7
Acid insoluble	11.6	8.2	12.5
Acid soluble albumins ..	1.18	0.7	1.50
Globulins	0.32	0.0	0.24
Potato powder	9.5	12.8	21.0

In the first two experiments, a one-hour absorption period resulted in essentially the same distribution of activities among the proteins, regardless of the 24-hour prewashing in the second case. But the portion of the total uptake accounted for by the proteins was somewhat less after the prewashing, perhaps because this process caused adsorption of Ca from the tap water on the cell walls, followed by simple exchange with Ca^{45} .

Table 4. *Activities of protein fractions after one hour absorption from 0.032—0.040 μ c P^{32} /ml. pH 5.8—6.4.*

Fraction	Exp. 15 (25° C)	Exp. 16 (2° C)	Exp. 17 (25° C)	Exp. 18 (2° C)
Mitochondria	617.0	29.6	112.0	14.2
Microsomes	900.0	58.5	131.0	16.1
Acid insoluble	555.0	19.1	105.0	9.0
Acid soluble albumins ..	5.8	0.5	2.0	0.8
Globulins	20.8	1.1	2.1	0.7
potato power	42.5	2.3	7.8	2.9

In all the experiments described so far, active absorption was apparently slight or non-existent. In order to determine the effect of active absorption on the results, a 24-hour prewashing was followed by a 24-hour absorption. Previous investigations (13) have shown these conditions to favour active absorption.

The distribution among the fractions was similar to that in the above two cases, though the differences were more pronounced and the total uptake was greater. Thus the three undialyzed fractions now accounted for 98.5 per cent of the protein activity, and the granules showed 10—15 times the activity of the acid-insoluble proteins. Yet the acid insoluble proteins in all three cases had about $10 \times$ the activity of the acid-soluble albumins.

d) *Uptake of P^{32} (Fractionation Method 2).*

In order to compare anion absorption with cation absorption, P^{32} was next investigated (Table 4). Again, the three undialyzed fractions accounted for nearly all the protein uptake, this time practically 100 per cent. The uptake at 25° C is again about 10—20 times that at +2° C. The activity per mg. was 10—20 times as high in the granules as in the potato tuber powder, and the acid-insoluble protein showed about $10 \times$ the activity of the albumins and globulins, at least at +2° C. At 25° C. this ratio was increased to 50—100 times.

Discussion

The results obtained by fractionation method 1 can be taken as showing little more than that absorption of Ca^{45} by potato slices is always accompanied by some uptake by the proteins. How much, and how it is distributed among the proteins cannot be revealed by this method since the frequent dialyses (and probably the $(NH_4)_2SO_4$ precipitation) permit large losses of adsorbed Ca^{45} . It is possible that the more stable albumins and globulins

withstand this treatment better than the other fractions, for they remain soluble (the former in water, the latter in salt solution). But the granules, at least, are known to be markedly altered by the nature of the solution in which they are dispersed (6). Due to the far superior technique used in fractionation method 2, discussion will be confined to the results obtained by this method.

In all cases, the granules proved to be the fractions that were responsible for the major accumulation by the proteins. This agrees with results of other workers (9). The somewhat greater activity of the microsomes than of the mitochondria may not be significant, since these »mitochondria» contained about 30 per cent starch-filled plastids (7). That it was a true accumulation is shown by the fact that the activity of the granules was in all cases somewhat greater than the activity of the potato tuber powder, and when active absorption was permitted to take place for 24 hours, it reached nearly 10 times as high a value. This increased activity due to active absorption may be explained in two ways: a) More granules are able to accumulate the ions. b) When active absorption occurs, the granules are much more active accumulators than when simple adsorption is primarily involved.

Any reliable explanation of these results awaits further investigations. The following is proposed as a working hypothesis. During the adsorption phase of ion accumulation, ions are exchanged at the surface of the protoplast, the number of exchanges depending on the number of loci available and the latter depending on respiratory energy. Thus respiration is involved even in the adsorption phase, though possibly the »ground respiration» is capable of supplying this energy. This adsorption may possibly occur at the surface of granules that happen to be located at the surface of the protoplast. At low temperatures and under any other conditions that stop cytoplasmic streaming, the accumulation process goes only this far. When cytoplasmic streaming occurs, these granules at the surface of the protoplast move into the cytoplasm along a stream together with their captured ions, and they are replaced at the surface by fresh granules which in their turn capture more ions. Thus the ions are distributed throughout the cytoplasm and may be released to other components of the cytoplasm, or if the granules move to the tonoplast, the ions may be released into the vacuole. Similar explanations have already been proposed by others (1).

The high activity of the acid-insoluble protein may perhaps be secondary, due to transfer from the granules. But this can only be decided by shorter absorption times. With regard to Lundegårdh's suggestion that nucleotides may be involved, it must be remembered that the granules contain RNA (4).

Summary

1. Uptake of Ca^{45} by potato tuber slices was determined at $+2^\circ \text{C}$ and $+25^\circ \text{C}$. The Q_{10} was nearly 3. Since the conditions were such as to preclude active accumulation, it is suggested that the number of adsorption loci increases with respiration rate.

2. Proteins obtained by fractionation method 1 (7) accounted for a very small but definite fraction of the total Ca^{45} accumulated. Quantitative measurements of the protein fractions and of the dialysate showed that large amounts of Ca^{45} were lost during dialysis.

3. Fractionation method 2, which avoids $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis for three of the five fractions, permitted a much greater recovery of the absorbed Ca^{45} in the proteins. The granules showed the greatest activity per mg. The three undialyzed fractions accounted for 90–99 per cent of the protein activity though comprising only 35–40 per cent of the proteins.

4. Active accumulation (for 24 hours vs. 1 hour in the above absorption experiments) resulted in the same distribution among the fractions though the differences were more pronounced and the total uptake greater.

5. Uptake of P^{32} resulted in essentially the same distribution of activities among the protein fractions as in the case of Ca^{45} .

6. A working hypothesis to explain these results is tentatively proposed.

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References

1. Arisz, W. H.: Contribution to a theory on the absorption of salts by the plant and their transport in parenchymatous tissue. — *Proc. Ned. Akad. Wet.* 48:420. 1945.
2. Arnon, D. I., Fratzke, W. E., & Johnson, C. M.: Hydrogen ion concentration in relation to absorption of inorganic nutrients by higher plants. — *Plant Physiol.* 17: 515. 1942.
3. Brooks, S. C.: Selective absorption with reference to ion exchange by the protoplasm. — *Trans. Far. Soc.* 33: 1002. 1937.
4. Claude, A.: Distribution of nucleic acids in the cell and the morphological constitution of cytoplasm. — *Biol. Symp.* 10: 111. 1943.
5. Davson, H. & Danielli, J. F.: The permeability of natural membranes. — Univ. Press, Cambridge. 1943.
6. Harmon, J. W.: Studies on mitochondria II. The structure of mitochondria in relation to enzymatic activity. — *Exp. Cell. Res.* 1: 394. 1950.
7. Levitt, J.: Two methods of fractionating potato tuber proteins and some preliminary results with dormant and active tubers. — *Physiol. Plant.* 5: 470. 1952.

8. Lundegårdh, H.: Absorption, transport and exudation of inorganic ions by the roots.
— Ark. f. Bot. 32 A: 1. 1945.
9. Mullins, L. J.: Radioactive ion distribution in protoplasmic granules. — Proc. Exp. Biol. and Med. 45: 856. 1940.
10. Osterhout, W. J. V.: The protoplasmic surface in certain plant cells. — Trans. Far. Soc. 33: 997. 1937.
11. Overstreet, R., Broyer, T. C., Isaacs, T. L., & Delwiche, C. C.: Additional studies regarding the cation absorption mechanism of plants in soil. — Amer. J. Bot. 29: 227. 1942.
12. Robertson, R. N.: Mechanism of absorption and transport of inorganic nutrients in plants. — Ann. Rev. Plant Physiol. 2: 1. 1951.
13. Stiles, W. & Skelding, A. D.: The salt relations of plant tissues. I. The absorption of potassium salts by storage tissue. — Ann. Bot. 4: 329. 1940.
14. Truog, E. (editor): Mineral nutrition of plants. — Univ. of Wisc. Press. 1951.

